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Reconstitution of intercompartmental protein transport in yeast extracts

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Reconstitution of Intercompartmental Protein Transport in Yeast Extracts

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Reconstitution of Intercompartmental Protein Transport in Yeast Extracts

David Baker

Abstract

Transport of α-factor precursor from the endpolasmic reticulum to the Golgi complex was reconstituted in yeast extracts. Transport is measured through the coupled addition of outer-chain carbohydrate to (^{35}S)methionine-labeled α-fator precursor translocated into the endoplasmic reticulum during a low temperature incubation. The reaction is absolutely dependent on ATP, stimulated 10-fold by cytosol, and occurs between physically separable sealed compartments. Sec mutations which block transport in vivo also block transport in vitro. Complementation of sec mutations in vitro provides a functional assay for the purification of individual intercompartmental transport factors.

The 21kd GTP binding Ypt1 protein (Ypt1p) is required for ER to Golgi transport in vitro. Fractions prepared from ypt1-1 mutant cells are defective in transport and anti Ypt1 Fab fragments inhibit the wild type transport reaction. Furthermore, immunodepletion of Ypt1p from wild type fractions blocks transport. Supplementation with a large Ypt1p containing particle restores transport to immunodepleted reactions Although the in vitro transport reaction requires physiological concentrations of calcium, Ypt1p functions independently of calcium in transport. First, buffering the free calcium at concentrations ranging from 10^{-9} to 10^{-5}M does not relieve inhibition by Ypt1 antibodies. Second, consumption of an intermediate that accumulates in calcium deficient incubations is not inhibited by anti-Ypt1 antibodies.

Randy Schekman

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Chapter 1

Introduction

A eukaryotic cell contains approximately ten billion protein molecules of ten thousand different types non uniformly distributed among approximately ten subcellular compartments. The probability of the observed partitioning of proteins occurring by random chance is about 1 in 10^{50000}. The generation and maintenance of the complex spatial organization of eukaryotic cells requires a sophisticated localization machinery that overcomes entropic barriers through the expenditure of chemical bond energy.

The basic operating features of the localization machinery are partially understood. Proteins contain structural motifs or "signals" which specify a particular subcellular location. Almost all proteins are synthesized in the cytoplasm and thus entry into a membrane enclosed compartment requires translocation across a lipid bilayer. In principle, protein localization could be accomplished by specific recognition of the signal by a receptor on the surface of the target compartment together with a mechanism for vectorially translocating the protein across the membrane. Such a mechanism suffices in prokaryotes and is used in eukaryotes in transport to organelles such as the nucleus (Kalderon et al., 1984), mitochondrion (Van Loon et al., 1986), and chloroplast (Smeekens et al., 1986). However, transport to the extracellular space and to the lysosome or vacuole, with several notable exceptions (Kuchler et al., 1989), does not proceed through such a "direct translocation" mechanism. Instead, proteins are routed through two specialized organelles, the endoplasmic reticulum (ER) and the Golgi complex.

This indirect route has several advantages. First, newly translocated proteins can be protected prior to exposure to the harsh environments of the extracellular space and the lysosome. Translocation is thought to involve the passage of proteins through an aqueous pore (Gilmore and Bloebel, 1985) in relatively extended, unfolded conformations (Eilers and Schatz, 1986) which can be exceedingly protease sensitive (Ostermann et al., 1989). Specialized enzymes in the ER promote the proper folding of newly translocated proteins (Freedman 1987; Lang et al., 1987), the assembly of multisubunit complexes, (Gething et

al., 1986) and catalyze post- translational modifications such as glycosylation which may enhance protein stability (Schlesinger and Schlesinger, 1987). Second, internal compartmentalization provides specialized microenvironments in which proteins can be subjected to elaborate postranslational modifications. Many hormones are synthesized as large precursors which mature in the Golgi complex through the action of a series of highly specific processing enzymes (Julius et al., 1984a,b; Herbert and Uhler, 1983). The complex carbohydrate structures found on many secreted proteins result from the sequential action of enzymes contained in four distinct subcompartments; the ER and the cis, medial and trans cisternae of the Golgi complex (Kornfeld and Kornfeld, 1985). Third, in cells exporting large amounts of protein, the surface area of the plasma membrane may not be large enough to accomodate sufficient numbers of translocation pores and processing enzymes. Internal organelles such as the ER can have many fold the surface area of the plasma membrane and thus a much higher potential translocation and processing capacity.

The path taken by newly synthesized proteins destined for the extracellular space was first defined by Palade and coworkers. Autoradiography and cell fractionation were used to follow pulse labeled secretory proteins through a series of membrane bounded organelles (the ER and the Golgi) identified by morphology and their constituent marker enzymes (Palade, 1975). These studies further showed that secretory vesicles are intermediates between the Golgi complex and the plasma membrane. Transport between compartments at each stage of the pathway is currently thought to be mediated by small transport vesicles (Pfeffer and Rothman, 1987). Newly synthesized proteins are translocated into the ER and then packaged into transport vesicles which eventually fuse with the cis Golgi cisterna. Small vesicles mediate transport from the cis to the medial and then to the trans Golgi cisternae (Orci et al., 1986). Proteins then pass through the trans Golgi network where they are packaged into vesicles bound for the plasma membrane or the lysosome (Griffiths and Simons, 1986).

The mechanisms underlying vesicular transport are poorly understood. There are three general physical problems: vesicle formation, vesicle targeting, and vesicle fusion. I will discuss each of these problems in turn, outlining the major unresolved questions and describing work from relevant model systems.

Vesicle formation

The first problem is to understand the forces which mechanically distort a locally flat lipid bilayer to form a 50-100nm vesicle. Curvature is opposed by membrane bending resistance (Evans, 1980) and the partial loss of hydration energy due to disruption of the ordered surface layer of interacting water molecules (Rand, 1981). Membrane vesicularization can result from tearing and resealing, but such a process necessarily involves leakage of membrane contents. Orderly vesicle formation requires a force to overcome the above resistances and a mechanism for controlling the resulting deformation of the membrane. A variety of models can be imagined in which surface or internal pressure resulting from an increase in lipid or protein concentration provides the force, and a contractile gap in a cytoskeletal meshwork surrounding the membrane, the means of regulating vesicle size. The ability of in vitro systems to support efficient vesicle formation reactions (see below) argues against this class of models, since the very low rates of protein and lipid synthesis in vitro and the fragmentation of subcellular architecture upon homogenization would be expected to interfere with force generation and control of membrane deformation, respectively. A second class of models involves the assembly of a regular, spherical protein scaffold onto the membrane. Membrane curvature would be driven by energetically favorable interactions between scaffold proteins, and the size and shape of the vesicles determined by the geometric properties of the resulting lattice. Geometrical constraints on the formation of a closed surface from identical subunits limit the possible scaffold structures to the platonic solids and their close relatives. Protein subunits have limited bending flexibility and thus icosahedral structures, which require the

least distortion of a planar lattice, may be preferred. The possible structures with icosahedral symmetry that can be formed from similar but not necessarily identical subunits have been enumerated (in the context of virus assembly) by Caspar and Klug (1962).

Several well characterized vesicle formation reactions involve assembly of a protein scaffold with icosahedral symmetry. The scaffold molecules may be integral membrane proteins or soluble proteins which can bind tightly to the membrane surface. The position of the membrane attachment site relative to the curvature of the lattice determines whether the membrane of a forming vesicle will be inside or outside the protein scaffold. The budding of Semliki Forest virus (SFV) and other alpha viruses involves an integral membrane scaffold protein. SFV particles contain a membrane spanning spike glycoprotein and a nucleocapsid composed of a single protein species and an RNA molecule (Harrison, 1983). The spike glycoproteins form trimers which pack into a hexagonal lattice. Pentagonal elements are introduced into the lattice as a result of interaction with the nucleocapsid. This produces a curved surface which ultimately closes on itself to form a regular icosahedron with T=4 symmetry (240 monomeric units) (Harrison, 1983; Simons and Fuller, 1987).

Budding of Vesicular Stomatitis virus (VSV), Human Immunodeficiency virus (HIV), and other more complex enveloped viruses may involve assembly of a protein scaffold on what will be the inner surface of the vesicle membrane. VSV particles have a matrix (M) protein situated between nucleocapsid and membrane which is essential for virus budding, and may form an internal, regular lattice (Simons and Fuller, 1987). The HIV Gag precursor is a soluble protein which associates with the inner surface of the plasma membrane via an N-terminal myristate and then assembles into 100-120nm particles which bud from the cell surface. The HIV envelope glycoprotein is not required for particle formation since budding occurs in cells expressing only the viral gag protein (Gheyson et al, 1989).

In contrast, receptor mediated endocytosis involves a scaffold protein, clathrin, which assembles on what will be the outer surface of the vesicle membrane (Goldstein et al., 1985). Three legged clathrin "triskelions" are composed of three light chains and three heavy chains (Kirchhausen and Harrison, 1981). Endocytic vesicles have a coat made up of a polyhedral lattice of clathrin triskelions. In the presence of 100kd "assembly" proteins, isolated triskelions will assemble into regular polyhedral structures with 12 pentagonal and 8 hexagonal faces (Zaremba and Keen, 1983). The triskelions are arranged on the vertices of a stretched dodecahedron, rather than on the vertices of an icosahedron, which results in a structure that is more open and less rigid than virus shells.

A soluble scaffold protein binding to either the inner or outer surface of the vesicle may be preferable to an integral membrane scaffold protein in intercompartmental protein transport reactions. Both clathrin and SFV spike protein can pack into planar hexagonal lattices (Harrison and Kirchhausen, 1983; Harrison, 1983). Additional factors, such as the assembly proteins or the nucleocapsid, are required for the formation of regular closed polyhedra. Pentagonal elements must be introduced into the lattice during assembly as conversion of a hexagonal lattice requires large scale breaking and rejoining of lattice interactions. Spike glycoproteins will associate when their concentration in the membrane reaches a threshold level; at this point either irreversible hexagonal lattices or icosahedral buds will form. A soluble scaffold molecule is not subjected to this kind of irreversible commitment at high concentration. Membrane association provides an additional level of regulation: the protein can be recruited to the membrane from a large soluble pool when required for vesicle formation.

A second problem concerning vesicle formation is selectivity; a transport vesicle must contain only a specialized subset of the components of the donor compartment. The different subcellular compartments have distinct membrane and protein compositions and execute different functions. The intercompartmental transport machinery must efficiently

and rapidly differentiate between resident proteins and cargo. The budding of Vesicular Stomatitis virus provides an interesting example of selective packaging. The lipid composition of the viral envelope is virtually identical to that of the host cell membrane, but almost all host proteins are excluded. However, many heterologous viral glycoproteins and the host Thy-1p can be incorporated into VSV envelopes (Simons and Fuller, 1987). Selective packaging can thus occur even in very simple systems, in this case involving at most two proteins. A possible mechanism for selective packaging in this and more general cases is that membrane (G protein) and internal (M protein) lattices sterically exclude noninteracting proteins. Proteins which do interact with vesicle components can be included without sacrifice of energetically favorable interactions.

Maintenance of compartmental identity does not require that all proteins are directly recognized by the transport machinery. In principle, only a single "receptor" protein must be actively sorted for each independent location; sorting of all other proteins could then result from binding to the proper receptor. Two cases in which proteins bound for a common destination share a common signal have been well documented. First, many of the resident ER proteins required for the assembly and modification of newly synthesized secretory proteins terminate in the four amino acids KDEL (Munro and Pelham, 1987). Long term residence of this class of proteins in the ER could be assured by the selective exclusion of a "KDEL receptor" from transport vesicles or selective retrieval of such a receptor from the Golgi. Second, most proteins bound for lysosomes are specifically modified with mannose 6-phosphate on carbohydrate chains (Lang et al., 1984). A mannose 6-phosphate receptor which cycles between the Golgi and the lysosome is responsible for the proper localization of this class of proteins (Brown and Farquar, 1984; Geuze et al., 1985). In both cases, how the receptors themselves are properly localized is an open question.

The viral budding model may explain some features of ER to Golgi transport. Inhibition of protein synthesis blocks transport vesicle accumulation (Chris Kaiser,

unpublished observations). A complex of newly synthesized proteins may play the role of the nucleocapsid template for vesicle formation. The protein lattices on the inside and in the membrane of the vesicles may be relatively loose, allowing most proteins to slip inside. Transport from the ER would in a sense be a default option, consistent with experimental observation (Pfeffer and Rothman, 1987). However, some proteins are retained in the ER, notably the resident enzymes mentioned previously and improperly folded proteins (Adams and Rose, 1985; Gething et al., 1986). A simple size exclusion mechanism may operate here. Misfolded proteins form large aggregates (Doms et al., 1987) and a multivalent KDEL receptor could create large complexes of ER resident proteins. Retention of large protein complexes in the ER may be a simple consequence of steric exclusion from transport vesicle lattices. The relatively small size of ER to Golgi transport vesicles (50nm diameter) may be related to such a size exclusion mechanism.

Vesicle targeting

A vesicle must recognize the target organelle amidst a great excess of non specific membrane. In transport to the lysosome, for example, a vesicle must specifically interact with only 0.4% of total cell membrane surface area. Specific recognition may involve a high affinity protein-protein interaction analogous to that mediating viral recognition of target cells. Glycoproteins on the surface of reovirus, hepatitis virus and HIV bind to cell surface receptors with dissociation constants in the nanomolar range (Laskey et al., 1987). In vesicular transport, however, there is a tradeoff between specificity and transport factor recycling: accuracy increases and the recycling rate decreases with increasing binding affinities. Assuming that binding is diffusion controlled, the half time for release of viral glycoproteins from cell surface receptors is approximately 10 minutes. For comparison, an entire vesicular transport reaction in yeast probably has a half time of less than a minute. I will return to the problems of rate and accuracy later in connection with GTP binding proteins. Lipid composition may also be important in targeting; phosphatidylcholine but

not phosphotidylserine liposomes will specifically bind to Golgi membranes in permeabilized cells (Kobayashi and Pagano, 1988).

Vesicles could reach the target organelle by diffusion or by a more directed movement, perhaps along cytoskeletal "tracks". The diffusion coefficients of Ficoll and dextran particles in cytoplasm have been determined by fluorescence recovery after photobleaching. A simple calculation based on recently published data (Luby-Phelps et al., 1987) shows that a 50nm diameter Ficoll particle in random walk motion in cytoplasm will sample a sphere of radius 5um in 50 seconds and a sphere of 15um in 8 min. Assuming that a 50nm transport vesicle behaves in a roughly similar fashion, the t1/2 of 3min for transport of the α factor precursor through a 5um yeast cell (Julius et al., 1984; Payne and Schekman, 1989) and 30min for VSV G protein through a 50um tissue culture cell (Strous and Lodish, 1980) are consistent with random walk motion. This conclusion is supported by the observation that Golgi vesicles move freely and rapidly between distinct Golgi stacks in cell fusion experiments (Rothman et al, 1984). The search times calculated above may be overestimates as the weak Van Der Waals attraction between membrane bilayers could reduce vesicle targeting to an effectively two dimensional diffusion problem.

vesicle fusion

Transport vesicles fuse with the appropriate target compartment without leakage of their contents into the bulk medium. Fusion between protein-free phospholipid vesicles does not occur under physiological conditions (White et al., 1983). The major energetic barrier to spontaneous fusion is the work needed to dismantle the ordered shell of interacting water molecules bound to polar lipid headgroups (Rand, 1981). Fusion of liposomes does occur when the hydration barrier is lessened by divalent cations such as calcium, but it results in massive lysis of the vesicles (Stegman et al., 1987). Proteins are thus required to overcome the hydration barrier in a precise, non lytic manner.

The best understood protein "fusagen" is the influenza virus hemagglutinin (HA). HA contains two hydrophobic amino acid sequences; a conventional membrane spanning domain which anchors the protein to the viral membrane, and a "fusion peptide" at an amino terminus created by proteolytic cleavage during biogenesis of the virus (White et al., 1983). Solution of the X-ray crystal structure of HA trimers (Wiley, 1981) has provided a basis for speculation about the mechanism of fusion. The trimer is a 135Å spike composed of three globular domains which form a head region and a 70Å stem which connects the head region to the membrane spanning domains. The three hydrophobic fusion peptides are buried. HA is irreversibly activated by exposure to low pH which is thought to trigger a conformational change exposing the fusion peptides. The peptides may then insert into the target membrane. HA, with one end anchored in the viral membrane and the other in the target membrane, may promote fusion by facilitating dehydration or simply by holding the membranes in close contact. Theoretical studies suggest that spontaneous fusion between lipid bilayers in artificial systems can result from thermal fluctuations which bring $1um^3$ of the membrane surfaces in contact (Leikin et al., 1987). From this early intermediate, vesicle fusion proceeds spontaneously, driven by an increase in hydrophobic interaction. HA may function simply by increasing the rate of formation of this early intermediate.

Fusion events in the secretory pathway are probably more complex. HA promotes membrane fusion at the expense of an irreversible conformational change, while intercompartmental transport factors are presumably used more than once. A reversible covalent modification with a hydrophobic moiety may replace the irreversible exposure of the fusion peptide. A common feature of both viral and cellular fusion mechanisms is a requirement for proteins only on one of the two membrane surfaces. Protein free liposomes fuse with the Golgi in permeabilized cells in an ATP dependent, NEM sensitive reaction (Kobayashi and Pagano, 1988), and influenza virus will readily fuse with liposomes (Stegman et al., 1989). These observations suggest that transport vesicle

proteins may be more important in preventing incorrect fusion events than in promoting correct ones. Indeed, loss of the clathrin coat leads to massive aggregation of formerly coated vesicles (Gex-Fabry and DeLisi, 1984).

Vesicle fusion must proceed through intermediates which maintain a continuous barrier to mixing between internal and external solutions. Electrophysiological and morphological studies of the fusion of secretory vesicles with the plasma membrane have provided some insight into the structures of early intermediates in membrane fusion (Breckenridge and Almers, 1987). These studies have suggested that fusion begins with the formation of a "fusion pore", a narrow channel across the space separating the transport vesicle from the plasma membrane. The fusion pore forms the first connection of the vesicle lumen with the extracellular space and then dilates to allow release of vesicle contents. The pore has a conductance (230ps) similar to that of a gap junction. The earliest fusion event may thus be the formation of a structure similar to an ion channel.

As described above, viruses carry out efficient vesicle formation, targeting and fusion reactions. However, the reactions are mediated by disposable components, with unidirectionality ensured by a low energy final state. A membrane spike protein can only be used in the budding of one vesicle, the high affinity targeting interactions between viral glycoproteins and cell surface receptors are effectively irreversible on transport time scales, and the fusion reaction involves an irreversible conformational change. Similar basic mechanisms may be used in intercompartmental transport reactions, but they must be adapted to allow multiple use of transport factors. The cost of recycling is presumably paid by nucleotide hydrolysis. Use of viral mechanisms in vesicular transport would minimally require additional, nucleotide dependent mechanisms for releasing scaffold proteins from closed shells, regulating receptor-target binding affinities, and retrieving fusion proteins from target membranes.

Understanding the mechanisms underlying vesicle formation, targeting and fusion in intercompartmental transport reactions will require identification and characterization of the relevant transport factors. Two approaches, one genetic, the other biochemical, have been applied to the study of the protein chemistry of transport.

Genetic analysis of transport in yeast.

Genetics provides a powerful means to identify individual components required in complex reactions. Mutants defective in the biological process of interest can be obtained by a suitable screening or selection procedure. Molecular biological methods can then be used to isolate and characterize the corresponding genes and their products.

A large number of yeast secretory (SEC) mutants have been isolated that have temperature-sensitive defects in protein transport (Novick et al., 1980). Analysis of the phenotypes of these mutants has shown that the yeast secretory pathway is similar in design to that of mammalian cells. At the non permissive temperature, one group of mutants accumulates core glycosylated secretory enzymes and ER membrane while mutants acting later in the pathway accumulate highly glycosylated secretory enzymes in structures resembling Golgi stacks or in secretory vesicles (Novick et al., 1981; Esmon et al., 1981). The mutants which block transport between the ER and the Golgi have been divided into two groups based on morphology and genetic interactions (Chris Kaiser, unpublished observations). SEC12, SEC13, SEC16 and SEC23 are required for the formation of 50nm intermediate vesicles, while SEC17, 18 and 22 are required for fusion of the vesicles with the Golgi.

The putative transport factors encoded by these genes can be studied by DNA sequence analysis and immunolocalization using antibodies against fusion proteins produced in bacteria. Three genes required for ER to Golgi transport have been subjected to this kind of analysis. SEC 12 encodes a 52 kd protein which contains a single membrane spanning domain and resides in ER and Golgi membranes (Nakano et al., 1988). Sec18p is an 84kd

protein which appears to be partially membrane associated, although the sequence suggests a soluble protein (Eakle et al., 1988). Sec 23p is an 84kd protein that is peripherally associated with a rapidly sedimenting compartment (Hicke and Schekman, 1989).

It is difficult to obtain more detailed information about the function of Sec gene products using molecular biological methods alone. The deduced amino acid sequences of Sec12p and Sec23p do not exhibit significant homology to the sequences of any known proteins. Taken optimistically, this may imply that transport reactions involve novel biochemical mechanisms. However, investigation of novel biochemistry requires a functional assay.

Analysis of transport in cell free systems

Biochemical analysis of reactions proceeding inside intact cells is complicated by the plasma membrane barrier and the vast diversity of unrelated macromolecules. Reconstitution of the process of interest in a cell free system allows direct manipulation of reaction conditions and purification of the essential factors. An in vitro reaction with purified components is amenable to a detailed enzymological and structural analysis.

Several intercompartmental protein transport reactions have been reconstituted in cell free systems. The first transport reaction to be reconstituted was the transport of the VSV G protein between successive compartments of the Golgi in chinese hamster ovary (CHO) cell extracts. Transport of G protein from mutant donor Golgi stacks which lack a critical N-acetylglucosamine (GlcNAc) transferase to wild type acceptor stacks which contain the transferase is measured by the coupled addition of ^{3}H-GlcNac (Balch et al., 1984a). Transport absolutely requires a crude cytosol fraction, ATP and proteins on the surface of Golgi membranes. More recently developed cell free systems, which support transport steps including transport from the ER to the Golgi (Beckers et al., 1988), from endosomes to the trans Golgi network (Goda et al., 1988), and the fusion of endocytic vesicles (Mayolga et al., 1989), have similar basic features.

Sequential intermediates in the intra Golgi transport reaction have been identified using a combination of specific inhibitors and morphological analysis. Addition of the guanine nucleotide analogue GTPγS to an in vitro transport reaction causes accumulation of non-clathrin coated vesicles (Melancon et al., 1987), while pretreatment of Golgi membranes with the alkylating agent N ethylmaleimide (NEM) leads to an accumulation of uncoated vesicles (Balch et al., 1984b). Ordering experiments have supported the hypothesis that transport of VSV-G through the Golgi is mediated by (non-clathrin) coated buds which pinch off from a donor cisternae to form coated vesicles and are then uncoated prior to fusion with the acceptor cisternae. The recent purification of the coated vesicles (Malholtra et al., 1989) should allow identification and characterization of the coat protein(s).

Purification of transport factors by direct fractionation of crude cytosol has proven difficult, probably because of the large number of factors involved. Instead, progress has depended on the use of specific inhibitors to inactivate individual transport factors which can then be purified by complementation. A major success of this approach was the purification of an NEM sensitive fusion protein (NSF) on the basis of its ability to restore transport to NEM inactivated Golgi membranes (Bloch and Rothman, 1988). NSF is a peripheral membrane protein required for the fusion of transport vesicles.

Further study has revealed a convergence of the genetic and biochemical approaches to transport. Amino acid sequence analysis showed that NSF is homologous (50% amino acid identity) to the Sec18 gene product. NSF activity can be detected in yeast extracts, and the correspondence between NSF and Sec18 has been confirmed by the demonstration that yeast cells bearing the SEC18 gene on a multi copy plasmid contain elevated levels of NSF activity (Wilson et al., 1989). NSF is also required for transport from the ER to the Golgi (Beckers et al., 1989) and the fusion of endocytic vesicles (Diaz et al., 1989) in cell free systems. The finding that a common factor functions at many stages of the secretory pathway and in organisms as diverse as mammals and yeast supports the view that a similar mechanism underlies most intercompartmental protein transport reactions.

Another emerging common feature of intercompartmental protein transport reactions is the involvement of guanine nucleotide binding proteins. The connection between GTP binding proteins and transport will be the subject of the remainder of the introduction.

21kd GTP binding proteins and transport.

The family of 21kd GTP binding proteins is large, growing and ubiquitous. The combination of molecular biological screens for ras related genes and biochemical screens for low molecular weight 21kd GTP binding proteins (Kikuchi et al., 1988), has led to an almost exponential increase in the number of known ras related 21kd proteins over the last several years. Over 20 of these proteins have been identified and the total number may well exceed 50 (Frank McCormick, personal communication). 21kd GTP binding proteins have been identified across eukaryotic kingdoms; H-ras was first identified in mammals (Ellis et al., 1981), Ypt1, in yeast (Galwitz, 1983), and rho in the snail Aplysia (Madaule et al., 1985). Although a connection has been established between some 21kd GTP binding proteins and tumorigenesis, the normal physiological role of most of these proteins is not known (Barbacid, 1987).

Study of small GTP binding proteins in yeast has suggested that a subset of these proteins function in intercompartmental protein transport. The SEC4, YPT1, ARF1, ARF2 and SAR1 genes all encode 20-24kd ras related proteins that contain the GTP binding consensus sequence and bind GTP in vitro. Disruption of the SEC4, YPT1 or SAR1 genes is lethal, as is disruption of both ARF genes (Salminen and Novick, 1987; Wagner et al., 1987, Tim Stearns, personal communication, Aki Nakano, personal communication). Work described in the following paragraphs suggests that Sec4p functions at a late stage, and Arfp,Sar1p and Ypt1p at early stages, of the secretory pathway.

SEC4 was identified in a selection for transport defective mutants. Temperature sensitive sec4 mutant cells accumulate secretory vesicles at the non-permissive temperature

(Novick et al., 1980). Sec4p has been localized to the cytoplasmic surface of secretory vesicles and the plasma membrane where it presumably promotes vesicle fusion (Goud et al., 1988).

ARF (ADP ribosylation factor) was first identified as a cofactor required for efficient ADP ribosylation of the α subunit of G_S by cholera toxin (Schleiffer et al, 1982). There are two closely related yeast ARF genes and a single bovine gene. Disruption of the ARF1 gene causes accumulation of the secretory enzyme invertase with incomplete Golgi modification. Depletion of Arf2p in cells lacking Arf1p causes accumulation of invertase with ER modification. Arf protein has been localized by immunofluorescence to the Golgi complex in mammalian cells (Tim Stearns, personal communication).

SAR1 was identified as a gene which on a multicopy plasmid suppressed the ER-Golgi transport defect of the sec12 mutant. Although doubling the SAR1 dosage restores transport in a cell with a mutant copy of sec12, overexpression of SAR1 cannot compensate for complete lack of Sec12p. Thus, suppression probably involves interaction of the two proteins rather than a bypass mechanism. Sar1p appears to function directly in ER-Golgi transport as depletion of Sar1p leads to accumulation of secretory proteins in the ER (Aki Nakano, personal communication).

The YPT1 gene was first identified as a DNA sequence between the yeast tubulin and actin genes capable of encoding a ras related protein (Gallwitz et al., 1983). Numerous proposals for the function of Ypt1p followed the demonstration that YPT1 is essential for yeast cell growth. Early studies suggested roles for Ypt1p in microtubule organization, mitosis, sporulation and starvation response (Schmitt et al., 1986; Segev and Botstein, 1987). More recently, several observations have linked Ypt1p to regulation of intracellular calcium . High extracellular calcium concentration suppress the temperature sensitive growth defect of the ypt1-ts mutant (Schmitt et al., 1988). Furthermore, deletion of a calcium ATPase gene, PMR1, suppresses the lethality of the cold sensitive ypt1-1 mutant (Rudolph et al., 1989). Ypt1p has also been implicated in secretion. Both the ypt1-1 and

ypt1-ts mutations cause accumulation of intracellular membranes and block transport of the secretory enzyme invertase. At the non-permissive temperatures, invertase accumulates in the ER in the ypt1-ts mutant, and in the Golgi in the ypt1-1 mutant (Schmitt et al., 1988; Segev et al., 1988). Finally, a mammalian homologue of Ypt1p has been localized to the Golgi in mouse L cells (Segev et al., 1988).

It is striking that only one of these genes (SEC4) turned up in screens for transport defective mutants and that ARF and YPT1 were first identified for reasons having nothing to do with transport. The Sec mutant screens would not have picked up ARF because of the gene duplication and may have missed YPT1 and SAR1 either because they do not easily mutate to ts alleles or simply because the screens were not saturating. Because of their fortuitous origins, Arf and Ypt1 can be viewed as a sample of the population of 21kd proteins unbiased with regards to transport. If the sample is representative of the population, there may be many more 21kd GTP binding proteins involved in transport.

Biochemical characterization of cell free systems which reconstitute intercompartmental transport reactions has provided independent evidence that GTP binding proteins function in transport. As mentioned previously, the guanine nucleotide analogue GTPγS inhibits transport of VSV G protein between Golgi cisternae (Melancon et al., 1987). Furthermore, GTPγS blocks fusion of endocytic vesicles (Mayolga et al., 1989) and the recycling of mannose 6-phosphate receptors to the trans Golgi (Goda and Pfeffer, 1988). Although inhibition of transport in several cases requires the presence of cytosolic factors (Melancon et al., 1987), the GTP binding proteins that are the targets of GTPγS inhibition have not yet been identified.

How might these proteins function in transport? The mechanistic trademark of the larger GTP binding proteins whose function is well understood is a cycle between two alternate conformations driven by GTP hydrolysis. The first major paradigm is provided by the G proteins of signal transduction. As secretion is constitutive in yeast a signal transduction role for the 21kd proteins seems unlikely, although such proteins could

perhaps transmit a fusion signal upon docking of a vesicle with the correct acceptor compartment. Alternatively, a group of signal transducers could be responsible for maintaining an ion gradient such as the pH gradient in the late secretory pathway (Mellman et al., 1986) which may be important in targeting.

A more natural analogy is to the GTP binding proteins of translation (Bourne, 1988; Baker et al., 1988). Translation and transport both involve the creation of a highly ordered state through a reiterated process. There are three basic requirements. First, the thermodynamics of the reactions must be such that they are essentially irreversible. Second as both translation and transport are probably close to limiting for growth, the reactions must occur at a reasonable rate. Third, the reactions must be accurate; errors in translation result in mutant proteins and errors in transport, in a breakdown of compartmentalization.

GTP binding proteins may be most important in increasing the rate and accuracy of biosynthetic reactions. Peptide bond formation from amino acyl (aa) tRNA's has a ΔG of -7kcal (Spirin, 1988) and template directed elongation can proceed in the absence of elongation factors and GTP (Gavrilova et al., 1976). Transport ATPases (NSF?) could play the role of aatRNA synthetases and provide the energy input to ensure unidirectionality. Although not required for unidirectionality, the elongation factors increase the rate of translation over 100 fold (Gavrilova et al., 1981). The slow step in a reiterated process can be recycling, the regeneration of starting conditions. This step may be sped by GTP hydrolysis; following the binding of an EfTu(GTP)--aatRNA complex to a ribosome, the GTP is hydrolyzed and the affinity of EfTu for the aatRNA and the ribosome drops 1000fold (Kaziro, 1980). A similar mechanism could speed the recycling of intercompartmental transport factors.

The most important role of EfTu in translation and by analogy that of transport GTPases may be to increase the fidelity of the process. Although template directed elongation can occur in the absence of EfTu, the error frequency is considerably higher (Gavrilova et al., 1976). Cognate and non-cognate aatRNA--codon interactions differ in affinity by less than

1000 fold. This would result in an unacceptably high rate of errors (> 1 in 10^3) if the GTPase activity of EfTu did not provide a double check mechanism.

An input of chemical energy can be converted to an increase in fidelity through a mechanism called "kinetic proofreading" (Hopfield, 1974). An initial binding equilibrium is followed by an irreversible conformational change (coupled to nucleotide hydrolysis) which in essence reduces the binding but not the dissociation rate constants. During the lag time before the next irreversible step, non-cognate complexes have a greater probability of dissociating than cognate complexes. The length of the lag time is independent of the complex present and thus provides an "internal kinetic standard" which can be set such that $1/k_{off}$ (non cognate) < lag time/ln2 < $1/k_{off}$ (cognate). Cognate pairs are thus selected over non-cognate pairs at two distinct steps, the initial binding equilibrium and the timed dissociation step. In the protein synthesis example, a new amino acid will be added to a growing polypeptide chain only if the aatRNA survives the two rounds of selection. Similar mechanisms could ensure the accuracy of vesicular transport reactions.

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The genetic dissection of transport in yeast and the biochemical analysis of transport in cell free extracts have complementary strengths and weaknesses. Yeast genetics has allowed the identification of individual transport factors, but the precise roles of these proteins in transport has remained obscure for lack of a biochemical assay. Reconstitution of transport in mammalian cell extracts has provided a biochemical assay for the function of transport factors, but the complexity of the reactions has made study of individual transport factors a difficult task.

Chapter 2 describes the development of a yeast in vitro transport assay which combines the strengths of the genetic and biochemical approaches to transport. The application of this system to the study of the role of Ypt1p in transport is described in Chapter 3. Additional information has been relegated to the appendices. Appendix 1 describes an

independent set of experiments which probe the role of clathrin in the uptake of the yeast pheromone α factor. Appendix 3 describes studies in progress which suggest that Ypt1 functions in association with a small vesicle or a large protein complex. Appendices 2 and 4 contain detailed technical information not included in the main text .

Chapter 2

Reconstitution of Sec Gene Product-dependent Intercompartmental Protein Transport

Summary

Transport of α-factor precursor from the ER to the Golgi apparatus has been reconstituted in gently-lysed yeast spheroplasts. Transport is measured through the coupled addition of outer chain carbohydrate to ^{35}S-methionine labeled α-factor precursor translocated into the ER of broken spheroplasts. The reaction is absolutely dependent on ATP, stimulated six fold by cytosol and occurs between physically separable sealed compartments. Transport is inhibited by the guanine nucleotide analog GTPγS. *sec23* Mutant cells have a temperature sensitive defect in ER to Golgi transport in vivo. This defect has been reproduced in vitro using *sec23* membranes and cytosol. Transport at 30^{o}C with *sec23* membranes requires addition of cytosol containing the *SEC23* (wild type) gene product. This demonstrates that an in vitro interorganelle transport reaction depends on a factor required for transport in vivo. Complementation of *sec* mutant defects in vitro provides a functional assay for the purification of individual intercompartmental transport factors.

Introduction

Newly synthesized proteins are directed to their proper locations through rapid and precise intercompartmental transport reactions. Secretory and plasma membrane proteins traverse a series of membrane enclosed compartments including the endoplasmic reticulum (ER) and the cisternae of the Golgi en route to the cell surface. While transport of proteins between these compartments has been extensively described in vivo (reviewed in Pfeffer and Rothman, 1987), the underlying mechanisms remain obscure. Reconstitution of intercompartmental transport in vitro is required to begin to study the enzymology of these reactions.

Several intercompartmental protein transport reactions have been reconstituted in mammalian systems. Reconstitution of transport between Golgi cisternae has led to the purification of at least one transport factor and identification of several novel transport intermediates (Melancon et al., 1987; Pfeffer and Rothman, 1987; J. Rothman, personal communication). Purification of transport factors has relied on specific inhibitors to inactivate single components followed by supplementation with untreated protein fractions. Direct fractionation has proved difficult, probably due to the large number of factors involved. Reconstitution of transport from the ER to the Golgi has recently been achieved using "semi-intact" Chinese hamster ovary cells which have lost most of their cytosol but retain intact organelles (Beckers et al., 1987).

A large number of yeast secretory (*sec*) mutants have been isolated which have temperature sensitive defects in protein transport (Novick et al., 1980). The motivation for developing an intercompartmental transport assay in yeast is the potential to purify and characterize individual transport factors through complementation of transport defective mutant reactions. Among the several stages in the yeast secretory pathway, protein transport from the ER to the Golgi is particularly amenable to analysis. Eleven complementation groups have been identified whose products are required for ER-Golgi

transport in vivo (Novick et al., 1981; Newman and Ferro-Novick, 1987). Furthermore, the addition of outer chain carbohydrate to core oligosaccharides serves as a convenient diagnostic of arrival in the Golgi (Esmon et al., 1981) (see Figure 1). A yeast ER-Golgi in vitro transport reaction has been previously reported (Haselbeck and Schekman, 1986), but a lack of cytosol dependence and low transport efficiency (<2%) has precluded effective exploitation of the mutants.

Here we report a new ER-Golgi in vitro transport reaction in which nearly one-third of the core glycosylated transport substrate receives outer chain carbohydrate. Two innovations were essential: a method for preparing semi-intact yeast and a method for following transport in extracts prepared from wild type cells. The reaction is absolutely dependent on ATP, stimulated six fold by cytosolic proteins and occurs between physically separable compartments. The reaction is blocked by GTPγS and depends on the *SEC23* gene product.

Materials and Methods

Materials

The yeast strains used in this study were GPY59 (*leu2*-3, -112, *ura3*-52, *trp1*-289, *prb1*, *pep4*::*URA3*, *gal2*, *MATa*), GPY60 (*leu2*-3, -112, *ura3*-52, *his4*-579, *trp1*-289, *prb1*, *pep4*::*URA3*, *gal2*, *MATα*), DBY5-3A (*sec23*, *leu2*-3, -112, *ura3*-52, *pep4*::*URA3*, *MATα*), and LHY3-8C (*sec23*, *leu2*-3, -112, *ura3*-52, *his3*, *MATα*). pDJ100 was obtained from D. Julius and J. Thorner. pSEC23 contains the *SEC23* gene on a 5.5 kB PvuII fragment inserted into the centromere vector YCp50. Casamino acids medium is 6.7 g/l yeast nitrogen base without amino acids (Difco Laboratories Inc.), 1% vitamin assay casamino acids (Difco Laboratories Inc.), 0.01% adenine, 0.01% histidine, 0.01% tryptophan, 0.01% methionine and 2% glucose with or without 0.002% uracil. YP

medium contained 1% Bacto-Yeast extract and 2% Bacto-peptone (Difco Laboratories Inc.). Cultures were grown in YP + 5% glucose (YPD) to early log phase as described (Deshaies, 1987). *SEC* strains were grown at 30°C and *sec* strains, at 24°C. One OD_{600} U was approximately 10^7 cells.

Anti α1→6 Man serum was produced in response to *mnn1 mnn2* cells as described (Ballou, 1970). α1→6, α1→2, and α1→3- mannose oligosaccharides were provided by Clint Ballou (this Department). Lyticase was prepared as described (Scott and Schekman, 1980). Concanavalin A (ConA)-Sepharose, apyrase (grade VII) and GDP-mannose were obtained from Sigma. Protein A-Sepharose was from Pharmacia. Other reagents were obtained as described (Kepes, 1988).

Preparation of Transport Competent Membranes

Cells were grown to 2-4 OD_{600} U/ml in YPD, harvested by centrifugation (1000 x g, 5 min, 24°C), and resuspended at 50 OD_{600} U/ml in 10 mM DTT, 100 mM Tris-Cl, pH 9.4. After 5 minutes at 24°C, cells were harvested by centrifugation, resuspended at 50 OD_{600} U/ml in 0.7 M sorbitol, 0.75 x YP, 0.5% glucose, 10 mM Tris-Cl, pH 7.5, 20 U lyticase per OD_{600} U cells and incubated at 30°C until the OD_{600} of a 1:100 dilution in H_2O dropped to less than 10% of the initial value. Spheroplasts were harvested by centrifugation, resuspended at 5 OD_{600} U/ml in 0.7 M sorbitol, 0.75 x YP, 1% glucose and incubated with gentle shaking for 20 min at 30°C. Spheroplasts, which had resumed metabolism, were harvested by centrifugation (1000 x g, 5 min, 4°C), washed and resuspended at 300 OD_{600} U/ml in lysis buffer (400 mM sorbitol, 20 mM Hepes pH 6.8, 150 mM KOAc, 2 mM MgOAc, 0.5 mM EGTA) at 4°C. Aliquots (200 µl) of the spheroplast suspension were transferred to Eppendorf tubes and frozen in the vapors above liquid N_2 in a sealed ice bucket. After 45 minutes the tubes were transferred to a -85°C freezer. No loss in transport activity was detected during two months of storage.

When sec mutant strains were used, the temperature during spheroplast formation and regeneration was reduced to 24°C.

Preparation of ^{35}S-Methionine-Labeled Prepro-α-Factor

Transcription of the plasmid pDJ100 and translation of the mRNA in a yeast extract were according to standard procedures (Hansen et al., 1986; Moldave and Gasior, 1983) except that the translation extract was prepared by glass bead agitation of cells (Deshaies, 1988). Translations (2-5 ml) were desalted by gel filtration on a Sephadex G-25 column (5 ml resin per ml translation) equilibrated in reaction buffer (20 mM Hepes pH 6.8, 150 mM KOAc, 250 mM sorbitol, 5 mM MgOAc). Peak fractions were pooled and aliquots frozen in liquid N_2 and stored at -85°C. No significant loss of transport competence was detected over one month of storage.

Preparation of Cytosol

Wild-type cells (4000 OD_{600} U) grown at 30°C in YPD or *sec* mutant cells grown at 24°C in casamino acids medium were harvested by centrifugation and washed in reaction buffer at 4°C. Cells were resuspended in 2 ml of reaction buffer containing 1 mM DTT and 0.5 mM PMSF, 4 gm of glass beads were added and the cells lysed by ten 30 sec periods of agitation on a VWR Vortexer 2 at full speed. The homogenate was clarified by centrifugation at 3000 x g for 5 min and the supernatant fraction further centrifuged at 100,000 x g for 30 min. The resultant S100 fraction was either desalted by filtration on Sephadex G-25 equilibrated in reaction buffer, and then frozen in aliquots in liquid N_2, or aliquoted and frozen immediately. Filtered and unfiltered cytosol stimulated transport to the same extent and were stable over several months of storage at -85°C. Protein concentration ranged from 20 to 30 mg/ml.

In Vitro Transport Reaction

For each experiment an aliquot of gently-lysed yeast was thawed by immersion in a 25°C water bath and the required amount of membranes (20 µl per reaction) washed 3 times by brief (~10 sec) centrifugation in a Fisher microcentrifuge and resuspension in 1 ml of reaction buffer at 4°C.

One stage reactions contained 5 µl of prepro-α-factor translation product (150,000 TCA precipitable cpm), 60 µg additional cytosol, 50 µM GDP-mannose, 1 mM ATP, 40 mM creatine phosphate (CP), 200 µg/ml creatine phosphokinase (CPK) and 20 µl (original volume) membranes washed as above and resuspended in reaction buffer to bring the reaction volume to 25 µl. The GDP-mannose, ATP, CP and CPK were added from a 10X stock prepared in reaction buffer and stored in small aliquots at -85°C. After 45 minutes at 20°C, reactions were terminated by addition of 40 µl of Laemmli sample buffer and heated for five minutes at 95°C.

The first stage of two stage reactions contained 8 µl prepro-α-factor translation product, 50 µM GDP mannose, 1 mM ATP, 40 mM CP, 200 µg/ml CPK and 20 µl (original volume) membranes washed as above and resuspended in reaction buffer to bring the reaction volume to 25 µl. The reaction mix was incubated for 15 minutes at 10°C and the membranes washed two times by centrifugation and resuspension in reaction buffer at 4°C. A complete second stage incubation contained in a final volume of 25 µl: washed pro-α-factor-containing membranes, 80 µg cytosol, 1 mM ATP, 50 µM GDP-mannose, 40 mM CP, and 200 µg/ml CPK. Typically, 8-10 reactions were combined in one tube during the first stage and the washed pro-α-factor-containing membranes were aliquoted to individual tubes with various additions at the begining of the second stage incubation. After 45 min at 20°C the reactions were terminated by addition of Laemmli sample buffer and heated for five min at 95°C.

Immunoprecipitation

A 20 µl portion of a terminated reaction was mixed with 35 µl of a 20% vol/vol suspension of Protein A-Sepharose, 8-10 µl of anti α1→6-Man serum or 8 µl of anti α-factor serum, and 1 ml of IP buffer (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 15 mM Tris-Cl, pH 7.5) in an Eppendorf tube and rotated for 2 hours at room temperature or overnight at 4°C. The immunoprecipitates were collected by centrifugation and washed twice with 1 ml of IP buffer, twice with 1 ml of 2 M urea, 200 mM NaCl, 1% Triton X-100, 100 mM Tris-Cl, pH 7.5, once with 1 ml of 500 mM NaCl, 1% Triton X-100, 20 mM Tris-Cl, pH 7.5, and once with 1 ml of 50 mM NaCl, 10 mM Tris-Cl, pH 7.5.

The washed immunoprecipitates were analyzed either by electrophoresis and autoradiography or directly by scintillation counting. Immunoprecipitates to be analyzed by electrophoresis were heated at 95°C in Laemmli sample buffer and electrophoresed on an 11.25% SDS polyacrylamide gel (Laemmli, 1970). The gel was treated with Amplify after fixation, dried, and exposed to X-ray film. Immunoprecipitates to be analyzed by scintillation counting were heated for 5 min at 95°C in 150 µl of 2% SDS then transferred to 8 ml scintillation vials. Five ml of Aquasol (New England Nuclear) were added and the samples were counted in a scintillation counter. The background, typically around 100 cpm, was determined by counting immunoprecipitates of complete reactions that had been stopped by addition of Laemmli sample buffer at the start of the incubation, and was subtracted from all reported values.

For Con A precipitations, 10 µl of a terminated reaction and 30 µl of a 20% vol/vol suspension of ConA-Sepharose were added to 1 ml of 500 mM NaCl, 1% Triton-X-100, 20 mM Tris Cl, pH 7.5, and samples were rotated for 2 hours at room temperature or overnight at 4°C. The washes and subsequent analysis were as described for antibody precipitations. Background, determined as above, was typically around 300 cpm.

Fractionation

A one-stage reaction scaled up 8 fold, and a parallel mock reaction with desalted cytosol substituted for the prepro-α-factor translation product, were incubated for 45 min at 20°C and then each processed as follows: A 25 µl aliquot treated with 0.1% Triton X-100 and an untreated 25 µl aliquot were diluted to 200 µl with lysis buffer and centrifuged at 100,000 x g for 10 minutes. One hundred µl of the remaining reaction mix was diluted to 1 ml with lysis buffer and centrifuged for 10 minutes at 300 x g. The low speed pellet fraction (LSP) was saved and the supernatant fraction centrifuged for 5 min at 7600 x g. This medium speed pellet (MSP) was saved and the supernatant fraction centrifuged for 10 min at 100,000 x g to give the high speed pellet (HSP). An unfractionated sample and each of the pellet fractions were analyzed by anti α1→6-Man or ConA precipitation following solubilization in Laemmli sample buffer (complete reaction). Fractions from the mock reaction were used to measure protein (Markwell et al., 1978) and NADPH cytochrome c reductase (Kubota, 1977).

Electron Microscopy

Samples were fixed in a mixture of 0.5% paraformaldehyde, 2.5% glutaraldehyde and picric acid at pH 7.4 for 2 h, followed by post-fixing with 2% OsO_4 in 0.1 M cacodylate buffer for 2 h. Specimens were dehydrated and embedded in Araldite. Sections were stained with uranyl acetate and lead citrate and examined with a Phillips 300 electron microscope.

Results

Preparation of Gently-Lysed Yeast

We sought a method for preparing yeast lysates competent for ER to Golgi transport. Recently, two methods for preparing transport-competent, semi-intact mammalian cells were described. These methods take advantage of the monolayer growth habit of the cells and cannot readily be generalized to cells such as yeast which grow in liquid culture. We explored freeze-thaw lysis of spheroplasts as a means of cell breakage. Early studies indicated that the rate of freezing determined the extent of lysis. Slow freezing preserved spheroplasts intact; rapid freezing ruptured spheroplasts and internal organelles. We then varied the freezing rate aiming for a gentle lysis procedure that released cytosolic proteins but left organelles intact.

Briefly, actively metabolizing spheroplasts were washed and resuspended in 400 mM sorbitol, 150 mM KOAc, 2 mM MgOAc, 0.5 mM EGTA, 20 mM Hepes pH 6.8, frozen over liquid N_2, and stored at -85^{o}C until needed. After thawing, about 50% of the spheroplasts were broken as judged by electron microscopy (data not shown). Examples of the morphology of broken spheroplasts are presented in Figure 2B,C,D. Much of the cytoplasm was released (compare the density of ribosomes in Figure 2A to that in 2B, C and D), but major organelles such as nuclei and vacuoles were largely intact. Ribosome-studded membranes, likely to be endoplasmic reticulum, were observed (see Figure 2C, above the nucleus). An example of breaks in the plasma membrane through which most of the cytosol may have escaped is indicated in Figure 2B, upper right.

Design of Transport Assay

Glycosylation serves as a convenient diagnostic of protein translocation into the ER and transport to the Golgi. Core oligosaccharides are added to proteins in the ER and then elongated in the Golgi by addition of outer chain carbohydrate (Kukuruzinska et al., 1987)

(Figure 1). Carbohydrate addition may be detected by a shift in mobility on an SDS gel, by precipitation with Concanavalin A (Con A), a lectin which recognizes mannose-containing oligosaccharides, or by immunoprecipitation with an anti α1→6-Man-specific antibody which recognizes only outer chain epitopes (Esmon et al., 1981).

Initial attempts at establishing an ER-Golgi in vitro reaction in gently-lysed yeast were modeled on the previously published yeast ER-Golgi assay (Haselbeck and Schekman, 1986). Invertase was accumulated in spheroplasts of an ER-blocked *sec* mutant at the non-permissive temperature. Spheroplasts were frozen, thawed, and membranes were incubated with ATP and cytosol at the permissive temperature and monitored for addition of outer-chain carbohydrate to invertase. These attempts met with little success.

We were concerned that mutant defects that were conditional in vivo might be accentuated in vitro and potentially be irreversible. Therefore a transport assay using components prepared from wild type cells was developed. The low steady state level of proteins in transit through the ER of wild type cells precluded use of an endogenous substrate in an in vitro transport assay. We reasoned that the lack of substrate could be overcome by introduction of an exogenous, radiolabeled protein via translocation into the ER of broken spheroplasts. This approach had two further advantages: first, intact spheroplasts surviving the lysis procedure would produce no background in the reaction (the labeled substrate could not penetrate) and, second, since the substrate was the only labeled species in the reaction, it could be followed easily.

We chose prepro-α-factor, the precursor of the secreted mating pheremone α-factor, as a substrate. Transport in vivo is rapid and has been extensively characterized (Julius et al., 1984; Fuller et al., 1988). Prepro-α-factor is converted to 30 kD core glycosylated pro-α-factor in the ER then processed to mature pheromone after addition of outer chain carbohydrate in the Golgi. Most importantly, prepro-α-factor is efficiently translocated post-translationally into yeast microsomes (Rothblatt and Meyer, 1986; Waters and Blobel, 1986; Hansen et al., 1986). Thus we could prepare ^{35}S-methionine labeled prepro-α-

factor translation product in advance and avoid the complications of translation in the presence of freeze-thaw lysates (endogenous mRNA background, ^{35}S-methionine incorporation by unbroken spheroplasts).

Prepro-α-factor Added to Gently-lysed Yeast Is Converted to the 30 kd ER Form and to a More Slowly Migrating Form That Is Immunoprecipitable with anti α1→6-Man Serum

^{35}S-Met labeled prepro-α-factor was synthesized in a yeast lysate and the translation reaction was desalted to remove unincorporated ^{35}S-methionine and glycerol. Translation product, washed membranes, yeast cytosol, an ATP regenerating system, and GDP-mannose were incubated together at 20°C for 45 minutes. As seen in Figure 3, lane 2, roughly half of the prepro-α-factor added to the incubation was converted to the 30 kd ER form. Furthermore, about 25% of the pro-α-factor was converted to forms of heterogenous mobility that migrated more slowly than the ER form, and which were not present when SDS was added at the beginning of the incubation (Figure 3, lane 1).

In vivo, the Golgi species of pro-α-factor migrates as a high molecular weight, heterogeneous collection of outer chain glycosylated forms (Julius et al., 1984; Fuller et al., 1988). To determine whether the heterogeneous forms observed in vitro were due to mannose addition by Golgi mannosyl transferases, we used an antibody specific for α1→6 linked mannose. The antibody precipitated the species in a broad region beginning slightly above the 30 kd ER form (Figure 3, lane 4); forms between this region and core-glycosylated precursor probably lack sufficient α16 linked mannose to be precipitated. The antibody reacted specifically with α1→6 linked mannose added during the incubation: no precipitation was observed when SDS was added at the beginning of the incubation (Figure 3, lane 3) or when competitor α1→6 linked mannose oligosaccharide (100 μM) was present during the immunoprecipitation. α1→2 and α1→3 linked mannose

oligosaccharides at millimolar concentrations did not inhibit immunoprecipitation (data not shown).

To avoid the necessity of running SDS gels after each experiment and to obtain more quantitative data, the amount of α-factor precursor receiving carbohydrate modification was determined by precipitation with anti α1→6-Man serum or ConA, followed by scintillation counting. ConA precipitation measured the total amount of prepro-α-factor translocated during an incubation, since ConA reacted with all glycosylated species (Figure 3, lane 6). The ratio of anti α1→6-Man precipitable pro-α-factor to ConA precipitable pro–α-factor, the "transport efficiency", was typically around 0.25 with some variation between different preparations and experiments. The anti α1→6-Man serum gave a slight underestimate of the amount of α-factor receiving outer chain modification, as it did not precipitate the portion of heterogeneous material just above the ER form.

Low Temperature Incubations Separate Transport from Translocation

Further characterization of the transport reaction required temporal separation of translocation and transport. ER to Golgi transport is blocked at low temperatures in other eukaryotic cells (Balch, 1986; Tartakoff, 1986). Therefore, we explored the effect of low temperature incubations on the production of core-glycosylated and outer chain-glycosylated precursor. As seen in Figure 4, translocation and α1→6-mannose addition were separated when the reaction was conducted at low temperature. At 10°C, translocation occurred readily but outer chain glycosylation was reduced to 10% of the 20°C value. Further characterization of the reaction supported the view that transport, not outer chain glycosylation per se, was the temperature dependent step (see below).

The selective block of transport at 10°C allowed study of transport independent of translocation through the use of two stage incubations. In the first stage prepro-α-factor translation product, washed membranes, an ATP regenerating system, and GDP-mannose were incubated together for 15 min at 10°C. The membranes were then washed and

warmed to 20°C with various additions. ConA precipitable cpm remained constant during the 20°C incubation indicating that no translocation competent precursor remained after the washes. In a typical experiment, membranes containing translocated precursor were subjected to varied conditions in a second stage to determine requirements of transport.

Properties of Transport

The kinetics of transport were investigated using a two stage reaction. Aliquots of the second stage incubation were stopped at invervals and anti α1→6-Man precipitable cpm were measured (Figure 5). A pronounced lag of about 10 min was followed by a 20 min linear phase which leveled off at 40 min. The lag period, which varied from 7 to 12 minutes between different experiments and preparations, may represent a rate-limiting early step in transport such as protein folding or vesicle budding.

ATP is required for transport from the ER to the Golgi in vivo (Novick et al., 1981; Balch et al., 1986). To determine whether ATP was required for the in vitro transport reaction, a two stage reaction was performed in which either an ATP regenerating system or apyrase (phosphoanhydride hydrolase) was added at the beginning of the second stage. ATP-depletion by treatment with apyrase (Figure 6), or glycerol with glycerol kinase (data not shown), completely blocked α1→6-mannose addition.

GDP-mannose is the sugar donor for outer chain addition (Kukuruzinska et al., 1987). The in vitro reaction was stimulated by, but did not require GDP-mannose (Figure 6), perhaps due to endogenous pools in the Golgi or de novo synthesis during the incubation.

Organelle integrity was required for and maintained during transport. Permeabilization of organelles by addition of the detergent saponin at the beginning of the second stage incubation completely blocked α1→6 mannose addition (Figure 6). Both the ER form and the heterogenously glycosylated form of the α-factor precursor resided within sealed compartments at the end of a transport reaction: both sedimented (Table 1A) and were protected from proteinase K (data not shown) in the absence, but not in the presence of

Triton X-100. The concentration of detergent required to release both α-factor precursor forms did not affect the sedimentation of an integral ER membrane protein, NADPH cytochrome c reductase (Table 1A), indicating that they were soluble within the lumen of their respective organelles.

Cytosolic proteins were required for transport. Transport was stimulated 6 fold by addition of cytosol in the second stage incubation (Table 2). Stimulation by cytosol reached saturation at 80 μg protein per reaction (data not shown). Stimulation was not due to a non-specific increase in protein concentration; 80 μg of BSA was without effect (Table 2). The residual transport occurring in the absence of added cytosol may be mediated by factors remaining associated with washed membranes.

Cytosol stimulation was due to proteins. Cytosol treated with trypsin or with the alkylating agent N-ethylmaleimide (NEM) failed to stimulate transport. Control treatments with trypsin inhibitor and trypsin or DTT and NEM had little effect on stimulation. Heat treatment also destroyed the stimulatory activity of cytosol (Table 2).

Anti α1→6 Man-Precipitable α-Factor is Enriched in a Compartment That Is Depleted of an ER Marker

The in vitro conversion of the ER form of the α-factor precursor to the anti α1→6 Man-precipitable form displayed the properties expected of an intercompartmental transport reaction. To address the intercompartmental nature of the reaction more directly we turned to subcellular fractionation. The yeast ER sediments more rapidly than the Golgi and the two organelles can be partially resolved by differential centrifugation (Esmon, 1986). We therefore investigated the sedimentation behavior of the α1→6 Man precipitable pro-α-factor relative to that of the majority of the ConA precipitable pro-α-factor.

A one-stage reaction was incubated for 45 min at 20°C and then fractionated into a LSP (low-speed pellet; 300 x g, 10 min) , MSP (medium-speed pellet; 7600 x g, 5 min) and HSP (high-speed pellet; 100,000 x g, 10 min) by differential centrifugation. Intact and

broken spheroplasts sedimented in the LSP while the MSP and HSP contained organelles that were released by the broken spheroplasts before or during the 20°C incubation. As can be seen in Table 1B, the LSP and MSP together contained 90 percent of the recovered NADPH cytochrome c reductase activity and 75 percent of the sedimentable core glycosylated pro-α-factor. In contrast, the α1→6-Man precipitable pro α-factor was recovered primarily in the HSP. We presume this fraction contains Golgi membranes, but the small scale of this reaction precluded direct assay of mannosyltransferase activity. The fractionation behavior of the highly glycosylated pro-α-factor was a property of the compartment that contained it: no sedimentation was observed when organelles were first permeabilized by addition of detergent (Table 1A).

Transport is Blocked by GTPγS

The G protein activators GTPγS and AlF_4 have been shown to inhibit the mammalian intra-Golgi in vitro transport reaction and cause the accumulation of coated transport vesicles (Melancon et al., 1987). As seen in Figure 7, GTPγS also inhibited the yeast ER-Golgi transport reaction. (This experiment was done by Michael Rexach). Half maximal inhibition occurred at about 5 μM. ATPγS and GMP-PNP had little inhibitory effect at concentrations up to 100μm. No inhibition was observed when 400μm GTP was added with the GTPγS. The combination of 4mM Fl^- and 50μM Al^{3+} reduced transport to 35% of control levels while neither compound alone had significant inhibitory effect.

Sec Mutations Block Transport in vitro

The sec mutants provide both a test of the authenticity of the in vitro reaction and a means to identify and purify individual transport factors. The analysis focused on the three ER blocked *sec* mutants (*sec12*, *18* and *23*) whose genes have been sequenced and gene products localized. We first examined the effects of imposing the *sec* mutant block in vivo. *sec* Mutant cell lysates were prepared as described in the Materials and Methods except that

spheroplasts were shifted to 37°C (the non-permissive temperature) during the last 15 min of the regeneration step. While this treatment had no effect on the transport capacity of wild type lysates, it completely blocked pro-α-factor transport in the mutant lysates (data not shown). Morphological alterations take place within *sec* mutants even after brief incubation at the non-permissive temperature (C. Kaiser and R. Schekman, unpublished results), thus defects revealed by an in vivo preshift may be indirect. To evaluate directly the role of *sec* gene products in the in vitro reaction, we analyzed the transport activity of mutant lysates prepared without exposure to the non-permissive temperature.

The temperature sensitive transport defect of *sec23* cells was reproduced in vitro. (This experiment was done by Linda Hicke). *sec23* Lysates were prepared from cells grown at the permissive temperature and ^{35}S-Met labeled prepro-α-factor was synthesized in a *sec23* translation extract. A two stage reaction was carried out using *sec23* components in the first stage. Cytosol added in the second stage was prepared either from *sec23* cells or from *sec23* cells containing a plasmid bearing the *SEC23* gene. Aliquots of each reaction were incubated at 15°C, 25°C or 30°C in the second stage. As seen in Figure 8, reactions that contained *SEC23* gene product proceeded efficiently at all temperatures. The reactions supplemented with *sec23* cytosol were markedly temperature sensitive. Transport at 30°C was reduced five fold relative to transport at 15°C. We could not test higher temperatures because the efficiency of the wild type reaction decreased rapidly above 30°C. *sec23* Cytosol did not inhibit reactions containing the *SEC23* gene product (data not shown). Although it is possible that there was a general temperature sensitive defect in *sec23* cytosol, the simplest explanation of these results is that transport at 30°C depended on the presence of the *SEC23* gene product.

We also examined transport in *sec12* and *sec18* lysates. *sec12* Lysates were defective in transport at all temperatures, even when supplemented with wild type cytosol. The lack of rescue was consistent with results from cell fractionation and sequence analysis, which show that the *SEC12* gene product is an integral membrane protein (Nakano et al., 1988).

sec18 Lysates were also defective at all temperatures. Supplementation with cytosol containing the *SEC18* gene product gave a two fold stimulation of transport (data not shown).

Discussion

We have developed a new yeast ER to Golgi in vitro transport assay. Prepro-α-factor, the precursor of the yeast mating pheromone α-factor, is synthesized in a yeast translation extract in the presence of ^{35}S-methionine and then translocated into the ER of gently lysed spheroplasts. Transport to the Golgi is measured through addition of outer chain carbohydrate. The amount of ^{35}S-Met-labeled prepro α-factor receiving outer chain is quantified by immunoprecipitation with an α1→6-Man specific antibody . Twenty five percent of the α-factor precursor that enters the ER is transported to the Golgi during a typical incubation.

The in vitro reaction has properties expected of intercompartmental protein transport. The reaction is dependent on energy, as is ER to Golgi transport in vivo, and addition of a soluble protein fraction stimulates the reaction six fold. Outer chain addition is accompanied by movement of the α-factor precursor from membranes that sediment rapidly to membranes that sediment more slowly and which are depleted of an ER marker enzyme. Transport is abolished by detergent, presumably due to a requirement for sealed compartments.

Artifactual signals may result from α1→6 mannose addition by a mannosyltransferase located in the ER or by nonspecific fusion between the ER and the Golgi. We consider these possibilities unlikely. First, secretory proteins may accumulate in the ER for over an hour in vivo without any detectable α1→6 mannose addition (Esmon et al., 1981). Second, the requirement for ATP and stimulation by cytosol are difficult to reconcile with either glycosylation in the ER or nonspecific fusion. Third, addition of α1→6 mannose in the ER and nonspecific fusion can not easily account for the observed differences in the sedimentation behavior of the modified and unmodified forms of the α-factor precursor. Finally, *sec* mutations which block transport in vivo block the in vitro reaction.

Role of GTP Binding Proteins in ER-Golgi Transport

YPT1, a gene encoding a GTP-binding protein, is required for transport of the secretory protein, invertase, from the ER to the Golgi in vivo (Segev et al., 1988; Schmitt et al., 1988). To investigate the role of YPT1 or other GTP binding proteins in transport, we have used the appoach of Melancon et al. who showed that the "G" protein activator GTPγS inhibits the mammalian intra-Golgi in vitro transport reaction. We find also that GTPγS specifically inhibits the ER-Golgi in vitro transport reaction. The inhibited component could function as a "G" protein like signal transducer. Alternatively, the GTPγS sensitive factor may play a role more analogous to that of the elongation factor Ef-Tu in protein synthesis (Kaziro, 1978). Targeting factors, which must recycle to direct repeated rounds of transport, may use the energy of GTP hydrolysis to cycle between two conformations as do many well characterized GTP binding proteins. More specifically, a GTP binding protein could guide transport vesicles to their destination in much the same way that Ef-Tu directs aminoacyl-tRNAs to the ribosome.

Sec Protein-dependent Transport in vitro

In vitro reconstitution has become the method of choice for studying the molecular mechanisms of interorganelle transport. Although reconstituted transport reactions share many of the properties of transport in vivo, there has been no direct demonstration that these in vitro reactions use any of the factors required for transport in vivo. In vitro analysis of the effects of *sec* mutations that block ER to Golgi transport in vivo provides an opportunity to authenticate the reaction.

A temperature sensitive defect in ER to Golgi transport caused by the *sec23* mutation has been reproduced in vitro. Transport at 30°C in *sec23* membranes supplemented with *sec23* cytosol is reduced five fold relative to transport at 15°C. Supplementation with cytosol containing the *SEC23* gene product restores transport at 30°C. Hence, the *SEC23* gene product is involved in transport in vitro as well as in vivo.

The sec mutants will facilitate purification and characterization of the proteins that mediate intercompartmental transport. Supplementation of mutant reactions with wild type cytosol provides a functional assay for the purification of *SEC* gene products. Molecular cloning techniques may be used independently to investigate the structure and location of Sec proteins. For example, the *SEC23* gene has been sequenced and the gene product localized using an antibody. The *SEC23* gene product is a relatively abundant cytoplasmic protein. In gently lysed yeast spheroplasts a large portion of the protein is loosely associated with the cytoplasmic surface of a membrane that sediments rapidly, though most of the protein is soluble under the conditions used to prepare cytosol for the in vitro reaction (Hicke and Schekman, unpublished results). Knowledge of the fractionation behavior of the *SEC23* gene product will be useful in developing models of the role the protein plays in transport and, together with the antibody, will aid purification of the functional *SEC23* gene product.

Further Applications of Protein Import into Gently-lysed Cells

Yeast spheroplasts can be broken by freeze thawing to produce ghosts which have lost most of their cytosol but retain intact organelles. These ghosts may support a variety of complex reactions in addition to the ones described here. The method is rapid and convenient: once frozen the spheroplasts can be stored indefinitely at -85^{o}C until needed. Since the procedure does not require adhesion of a monolayer of cells to a surface, it may be extended to a variety of cell types that grow in liquid culture.

The system we have developed may be amenable to reconstitution of later steps in protein transport. Intra-Golgi transport may be investigated by a simple extension of the ER-Golgi assay. The first step in the proteolytic maturation of the α-factor precursor, cleavage by the KEX2 endopeptidase, is thought to take place in a late Golgi compartment (Fuller et al., 1988). Under standard reaction conditions, we have detected the production of an α-factor related species that comigrates with mature α-factor on a 15%

polyacrylamide gel but thus far have been unable to demonstrate ATP and cytosol dependence (A. Capriotti, D. Baker, R. Schekman, unpublished results).

The use of a transport substrate synthesized in vitro allows considerable flexibility. In previous intercompartmental transport assays, the substrate has been accumulated in the donor compartment in vivo prior to lysis. This approach is limited to rapidly synthesized proteins that can be caught in transit through the donor compartment, proteins with conditional defects in transport, or in yeast sec mutant cells. Our approach is free of these restrictions. Any step along the secretory pathway may potentially be reconstituted by introducing an appropriate radiolabeled substrate into the ER of lysed spheroplasts. For example, the precursor to the yeast vacuolar protease carboxypeptidase Y may be used to follow transport to the vacuole. Furthermore, substrates that vary by the presence or absence of a sorting determinant may be introduced into lysed spheroplasts to investigate the molecular mechanisms of sorting.

Table 1. Fractionation of Compartments Containing Core Glycosylated and Outer-Chain Glycosylated Pro-α-Factor

Fraction	Protein (μg)	NADPH–Cytochrome c Reductase (% total)	Core Glycosylated α-Factor (% total)	Anti-α1→6-Man Precipitable α-Factor (% total)
A Total	557	100	100	100
– Triton pellet	361	88	77	85
+ Triton pellet	213	61	9	3
B LSP	183	57	26	7
MSP	54	24	17	12
HSP	55	9	15	41

A large one-stage reaction was fractionated as described in Experimental Procedures. (A) Two aliquots were centrifuged at 100,000 × g for 10 min after no treatment (– Triton pellet) or after addition of 0.1% Triton X-100 (+ Triton pellet). (B) A separate portion was fractionated into an LSP (300 × g, 10 min), an MSP (7600 × g, 5 min) and an HSP (100,000 × g, 10 min) by differential centrifugation. An unfractionated sample and each of the pellet samples were analyzed as described in Experimental Procedures. The values presented for core glycosylated pro-α-factor were obtained by subtracting the anti-α1→6-Man precipitable cpm from the ConA precipitable cpm. This represents an overestimate of core glycosylated material because not all outer-chain glycosylated pro-α-factor was precipitated by the anti-α1→6-Man serum. Material not accounted for in the pellet fractions may have been released into the soluble fraction during incubation at 20°C or during fractionation. Technical problems precluded quantitation of this material. Total values were 49,052 cpm of core glycosylated pro-α-factor and 15,424 cpm of anti-α1→6-Man precipitable pro-α-factor.

Table 2. Cytosolic Protein Is Required for Transport

Addition	Anti-α1→6-Man Precipitable cpm (% maximum)
Untreated cytosol (80 μg)	100
Reaction buffer	16
BSA (80 μg)	14
Treated cytosol (80 μg):	
+ Trypsin $\xrightarrow{30\ min,\ 4°C}$ + trypsin inhibitor	15
+ Trypsin + trypsin inhibitor $\xrightarrow{30\ min,\ 4°C}$	86
+ NEM $\xrightarrow{30\ min,\ 4°C}$ + DTT	13
+ DTT + NEM $\xrightarrow{30\ min,\ 4°C}$	83
15 min, 95°C	20

A 225 μl two-stage reaction mix was incubated for 15 min at 10°C, and the membranes were washed and resuspended to a final volume of 180 μl in reaction buffer containing GDP-mannose and an ATP-regenerating system. Portions (20 μl) were aliquoted to tubes containing 5 μl of the indicated protein solution. After 45 min at 20°C, 40 μl of Laemmli sample buffer was added, and reaction products were analyzed by immunoprecipitation followed by scintillation counting. BSA was dissolved in reaction buffer, and the cytosol was desalted by gel filtration in reaction buffer. For cytosol treatments, 250 μg/ml trypsin or 10 mM NEM was incubated with cytosol for 30 min at 40°C and quenched by addition of 500 μg/ml soybean trypsin inhibitor or 200 mM DTT. In control treatments, the trypsin inhibitor or DTT was added at the beginning of the incubation. For heat treatment, cytosol was incubated for 15 min at 95°C and then centrifuged for 1 min at 12,000 × g to remove aggregates of denatured protein.

Figure 1. Glycosylation Coupled to Translocation of Proteins into the ER and Transport to the Golgi

Core oligosaccharides are added to proteins upon translocation into the ER. Transport to the Golgi is blocked in the *sec* and *bet* mutants listed. In the Golgi, core oligosaccharides are extended by addition of outer chain carbohydrate. M, mannose; GNAc, N-acetylglucosamine; Asn, asparagine.

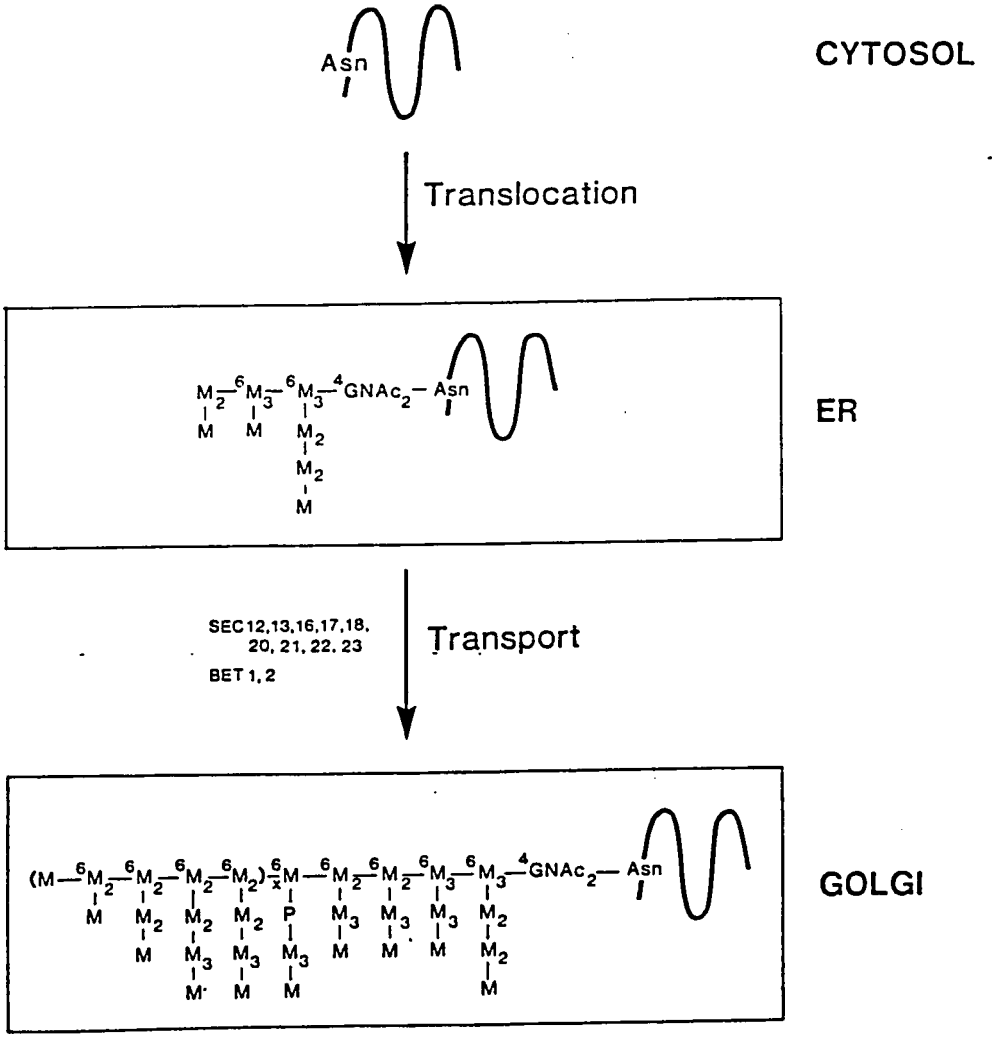

Asn
CYTOSOL
Translocation
ER
SEC12,13,16,17,18,
20, 21, 22, 23
BET 1, 2
Transport
GOLGI

Figure 2. Morphology of Gently-lysed Spheroplasts

Spheroplasts were processed for electron microscopy before (A) and after (B,C,D) freeze thawing. The arrow in B marks a break in the plasma membrane. N, nucleus; V, vacuole.

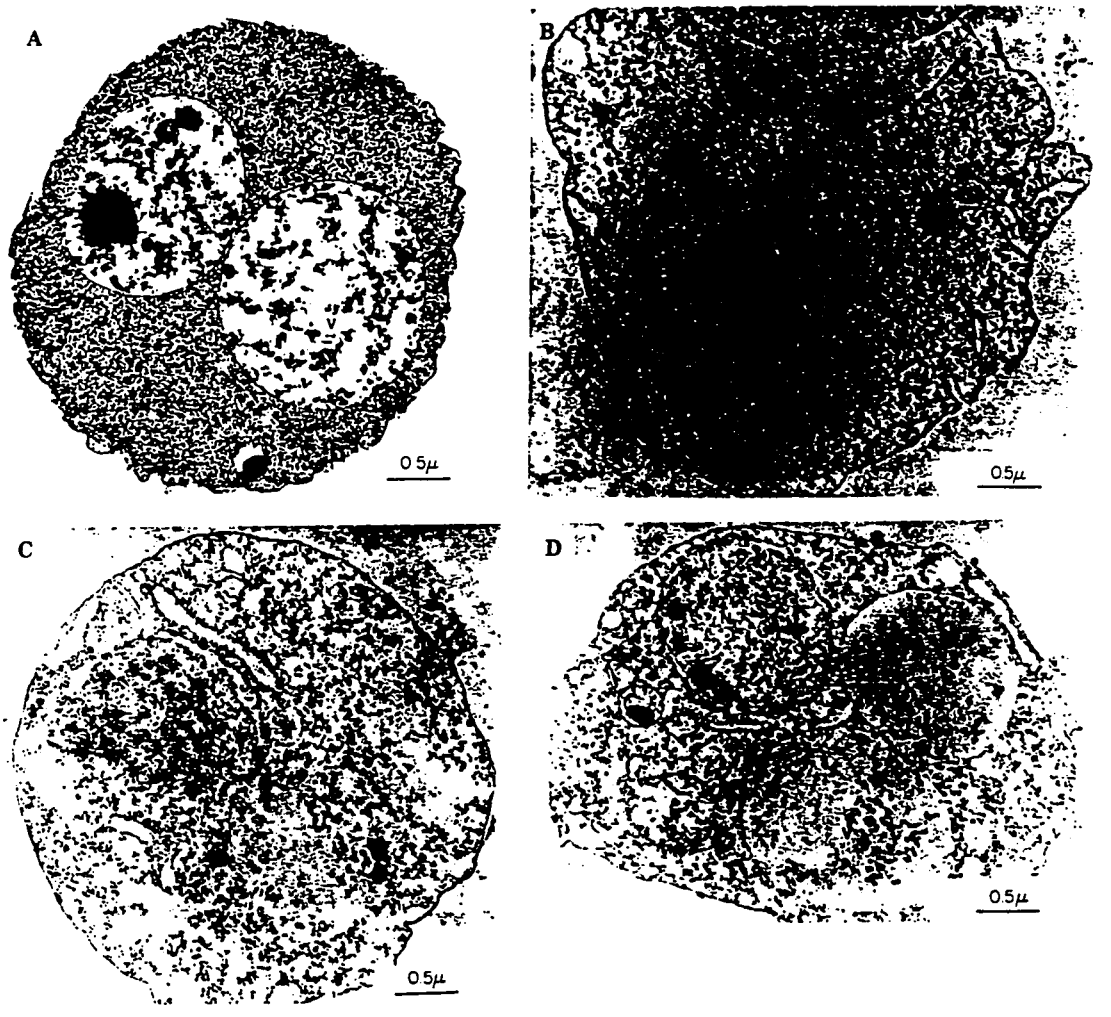

A
0.5μ
B
0.5μ
C
0.5μ
D
0.5μ

Figure 3. Prepro-α-factor Incubated with Gently-lysed Yeast Is Converted to the 30 kd ER Form and to a More Slowly Migrating Form That Is Immunoprecipitable with anti α1→6-Man Serum

Gently-lysed yeast and ^{35}S-methionine-labeled prepro-α-factor were incubated together in the presence of GDP-mannose and an ATP regenerating system. Laemmli sample buffer was added at the time indicated and the quenched reactions heated for 5 min at 95^{o}C. Portions (20 μl) of each reaction were precipitated with anti α-factor serum, anti α1→6-Man serum or ConA. The immunoprecipitates were electrophoresed on an 11.25% polyacrylamide gel. The gel was incubated with Amplify, dried, and exposed to X-ray film for 48 hrs.

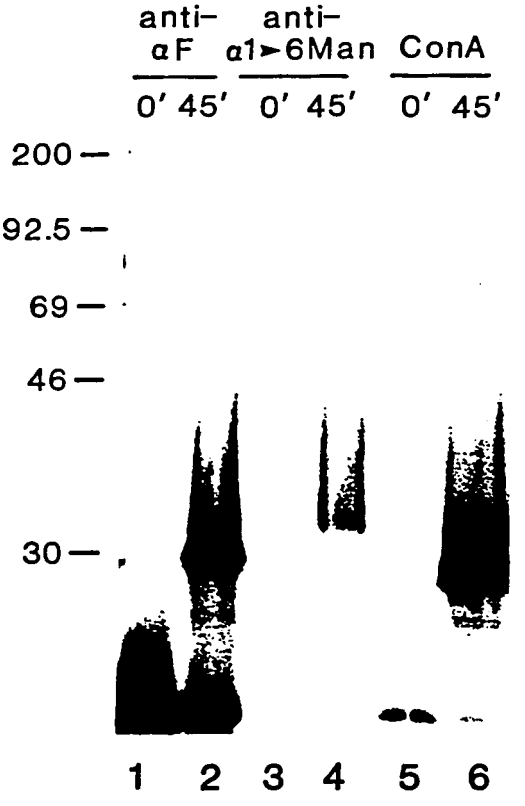

anti-
αF
anti-
α1►6Man
ConA
0′ 45′ 0′ 45′ 0′ 45′
200
92.5
69
46
30
1 2 3 4 5 6

Figure 4. Temperature Dependence

^{35}S-Methionine-labeled prepro-α-factor, gently-lysed yeast, an ATP regenerating system and GDP-mannose sufficient for seven one-stage reactions were mixed in one tube at 4°C. Aliquots (25 µl) were incubated for 45 min at the indicated temperature and then terminated by addition of 40 µl Laemmli sample buffer and heating at 95°C. Reaction products were precipitated with anti α1→6-Man serum or ConA and the precipitates analyzed by scintillation counting.

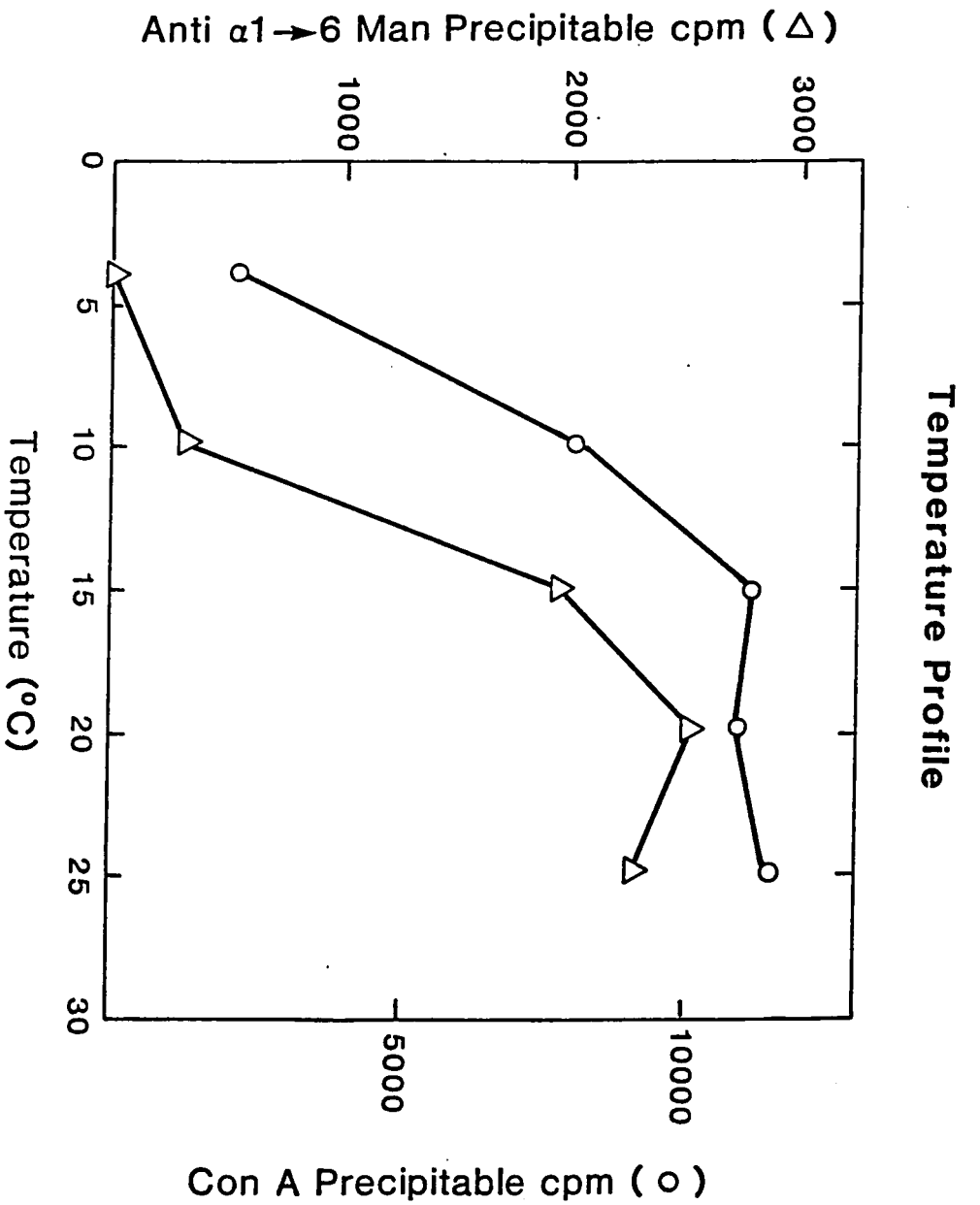

Anti α1→6 Man Precipitable cpm (Δ)
1000
2000
3000
0
5
10
15
20
25
30
Temperature (°C)
Temperature Profile
5000
10000
Con A Precipitable cpm (o)

Figure 5. Time Course of Transport

Aliquots were terminated at intervals after the beginning of the second stage of a two-stage reaction and analyzed by immunoprecipitation and scintillation counting. The zero time background was 120 cpm was subtracted from the presented values. ConA precipitable pro-α-factor averaged 4739 cpm per point.

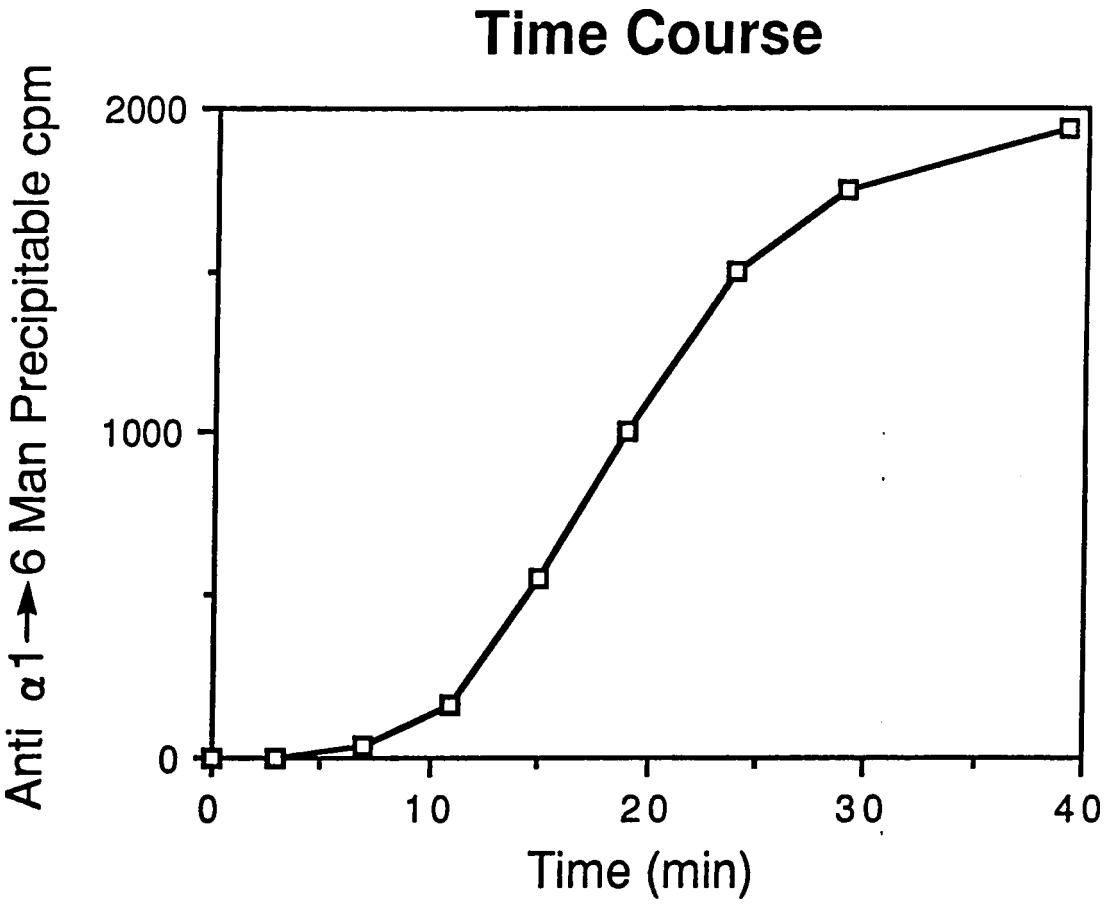

Time Course
Anti α1→6 Man Precipitable cpm
2000
1000
0
0
10
20
30
40
Time (min)

Figure 6. Transport is Energy Dependent, Stimulated by GDP-Mannose, and Inhibited by Detergent

At the end of the first stage (15 min $10^{o}C$ incubation) membranes containing translocated pro-α-factor were washed twice and aliquoted to tubes containing 50 μM GDP-mannose, an ATP regenerating system, and 0.2% saponin as indicated. The tube lacking ATP contained 2.5 units of apyrase. Samples were incubated for 45 min at $20^{o}C$ and then immunoprecipitated and counted. The background at the beginning of the second stage was 125 cpm and was subtracted from the reported values. ConA precipitable pro-α-factor averaged 5825 cpm per point.

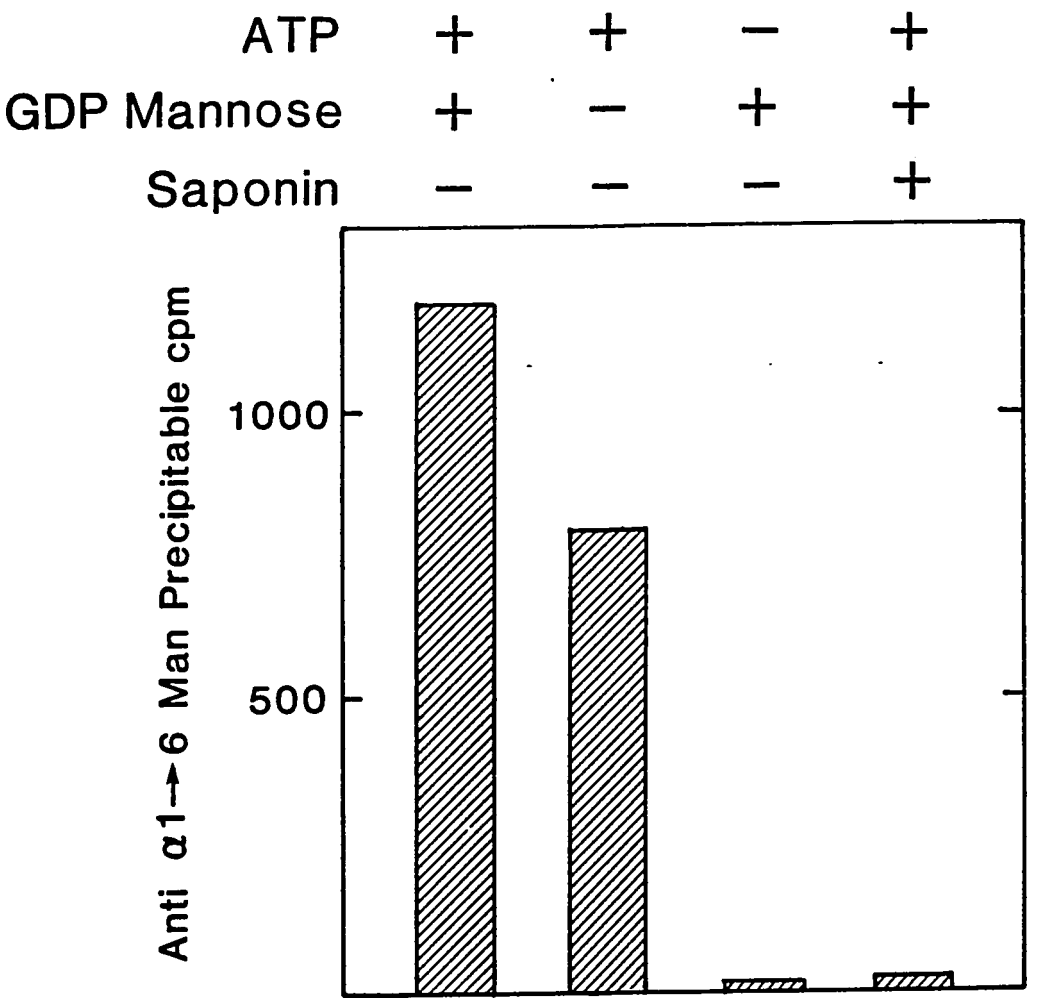

ATP + + − +
GDP Mannose + − + +
Saponin − − − +
Anti α1→6 Man Precipitable cpm
1000
500

Figure 7. Transport is inhibited by GTPγS

Increasing concentrations of GTPγS were added to aliquots of complete reaction mix at the beginning of the second stage of a standard two stage reaction. Samples were terminated after 45 min at 20°C and analyzed by immunoprecipitation and scintillation counting. ConA precipitable pro-α-factor averaged 6900 cpm per point.

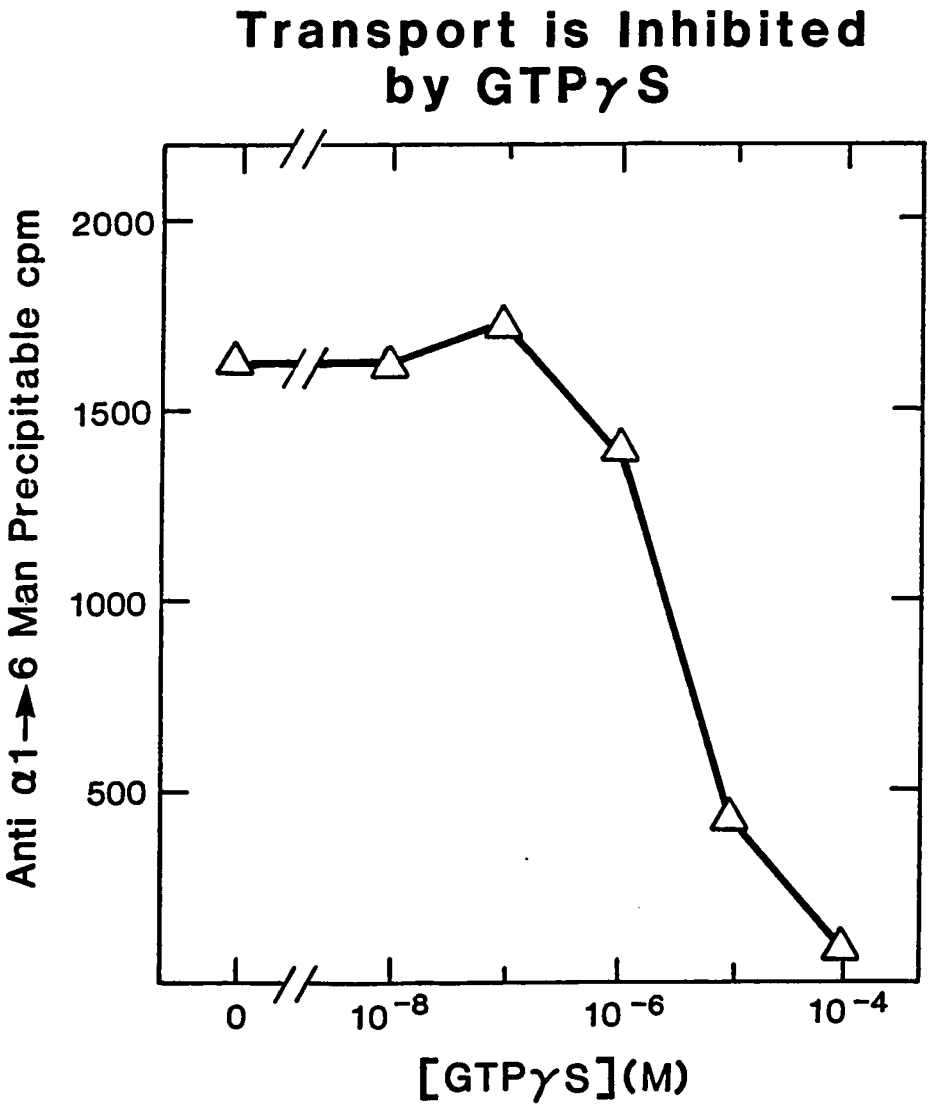
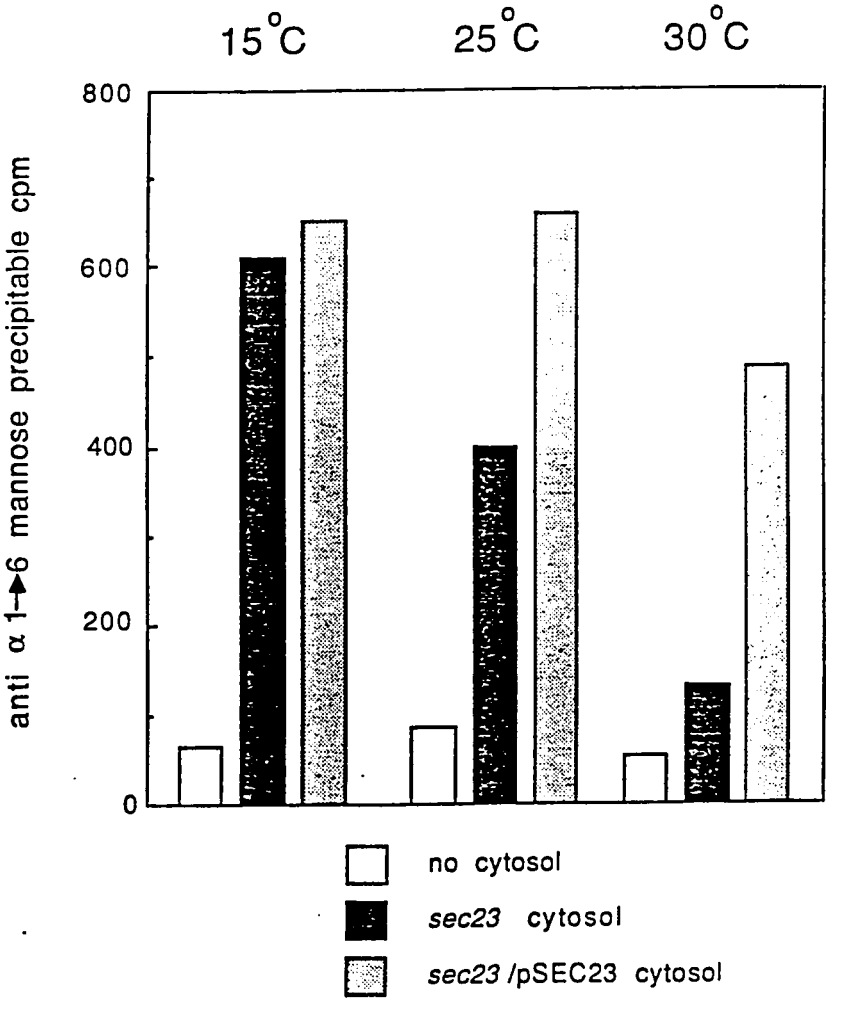

Transport is Inhibited
by GTPγS
Anti α1→6 Man Precipitable cpm
2000
1500
1000
500
0
10⁻⁸
10⁻⁶
10⁻⁴
[GTPγS](M)

Figure 8. In vitro Transport with Mutant *sec23* Components Is Temperature-sensitive

Broken spheroplasts prepared from a *sec23* strain were incubated with ^{35}S-prepro-α-factor made in a *sec23* translation extract during the first stage of a two-stage reaction. For the second stage, washed membranes were mixed with cytosol prepared from either a *sec23* strain or the same *sec23* strain carrying a *SEC23* gene on a single-copy plasmid (pSEC23). Transport was allowed to proceed at 15°C or 25°C for 45 min, or at 30°C for 25 min. ConA precipitable pro-α-factor averaged 4482 cpm per point.


15°C
25°C
30°C
800
600
400
200
0
anti α 1→6 mannose precipitable cpm
no cytosol
sec23 cytosol
sec23 /pSEC23 cytosol

Chapter 3

The GTP-binding Ypt1 Protein and Ca^{2+} Function Independently in a Cell-free Protein Transport Reaction

Summary

The 21kd GTP binding Ypt1 protein (Ypt1p) is required for protein transport from the endoplasmic reticulum to the Golgi complex in yeast extracts. Fractions prepared from ypt1-1 mutant cells are defective in transport and anti Ypt1 Fab fragments inhibit the wild type transport reaction. The in vitro transport reaction also requires physiological levels of calcium. However, Ypt1p functions independently of calcium. First, buffering the free calcium at concentrations ranging from 10^{-9} to 10^{-5} M does not relieve inhibition by Ypt1 antibodies. Second, consumption of a calcium-requiring intermediate that accumulates in calcium-deficient incubations is not inhibited by anti-Ypt1 antibodies although completion of transport requires ATP and an NEM-sensitive factor. Thus Ypt1p and Ca^{2+} are required in distinct transport partial reactions.

Introduction

Analysis of mutations in two yeast genes, *SEC4* and *YPT1* (Salminen and Novick, 1987; Segev et al., 1988) suggested that ras-like GTP-binding proteins might function in transport. *sec4* Mutant cells are deficient in the last step of secretion; mature secretory vesicles accumulate (Novick and Schekman, 1980). The Sec4 protein (Sec4p) is located on the cytoplasmic surface of such vesicles (Goud et al., 1988). In contrast, *ypt1* mutants accumulate endoplasmic reticulum (ER) and Golgi-like membranes indicating a defect early in the pathway (Segev et al, 1988; Schmitt et al, 1988). Immunolocalization studies in mammalian cells suggest the Ypt1 protein (Ypt1p) is associated with the Golgi complex (Segev et al, 1988).

The observation of a connection between GTP-binding proteins and secretion stimulated an examination of the effect of GTP analogues on protein transport in cell-free systems. The non-hydrolyzable analog GTPγS blocks vesicular transport within the Golgi complex (Melancon et al., 1987), transport from the ER to the Golgi (Baker et al., 1988; Ruohola et al., 1988; Beckers and Balch, 1989), and the fusion of endocytic vesicles and recycling of the mannose-6-phosphate receptor (Mayolga et al., 1989; Goda and Pfeffer, 1989); however the inhibition has not been associated with a specific protein target. Transport of the α-factor precursor from the ER to the Golgi has been reconstituted in yeast lysates (Baker et al., 1988; Ruohola et al., 1988). This reaction is inhibited by GTPγS; based on the *in vivo* work Ypt1p is a candidate target protein.

In addition to its role in secretion, Ypt1p has been implicated in other cellular processes (Schmitt et al., 1987; Schmitt et al., 1988; Rudolph et al., 1989). Two observations have suggested a link between *YPT1* and Ca^{2+}. High extracellular concentrations of Ca^{2+} suppress the temperature sensitive growth defect of the *ypt1*-ts mutant (Schmitt et al., 1988). Furthermore, deletion of *PMR1*, a gene that encodes a Ca^{2+}-ATPase homologue, suppresses lethality of the *ypt1-1* mutation (Rudolph et al., 1989).

Here we examine the role of Ypt1p and Ca^{2+} in the yeast ER to Golgi *in vitro* reaction. We find that either a mutation in *YPT1* or antibody against Ypt1p blocks transport. Further, we show that Ypt1p and Ca^{2+} function independently in transport.

Materials and Methods

Cells, Plasmids, and Materials.

S. cerevisiae strains used are: GPY60 (MATα, *ura3-52*, *trp1*, *leu2*, *his4*, *pep4:URA3*); NSY12 (MATα, *ura3-52*, *trp1*, *his4*, *lys2*, *leu2*, *pep4::URA3*, *ypt1-1*); DBY1034 *YPT1*; DBY1803 *ypt1-1*, (Segev and Botstein, 1987). Plasmid pRS9 was constructed by ligation of the EcoR1-BamH1 fragment of *YPT1* from pRB301 (14), into the Xba1 and BamH1 sites of the *trp* promoter in pHTH207-1 (a gift from Dennis Henner), using a 50 base pair oligonucleotide that includes the 5' end of the *YPT1* gene with Xba1 site. Ypt1p was purified from *E. coli* cells containing pRS9 by urea extraction of inclusion bodies (Desplan et al, 1985). Anti-TrpE-Ypt1 antiserum and affinity purified anti-Ypt1 and anti-trpE antibodies were prepared and checked as described (Segev et al, 1988). Anti-Ypt1 Fab fragments were prepared from anti-trpE-Ypt1 antiserum using papain coupled to agarose beads (Pierce) according to the manufacturer's instructions. Other materials were as described previously (Baker et al., 1988).

Preparation of Microsomes and High Speed Pellet (HSP).

Spheroplasts, prepared as described (Baker, 1988), were harvested by centrifugation through a cushion of 0.8 M sucrose, 1.5% ficoll 400, 20 mM Hepes, pH 7.4 (10,500 x g, 5 min, 4°C), resuspended at 100 OD_{600} U/ml in lysis buffer (100 mM sorbitol, 20 mM Hepes, pH 7.4, 50 mM KOAc, 2 mM EDTA, 1 mM DTT, 1 mM PMSF) at 4°C and homogenized with a motor-driven Potter-Elvejem homogenizer (10 strokes). After centrifugation of the lysate at 3000 x g for 10 min, the supernatant was collected and

centrifuged at 12,000 x g for 10 min. The resulting microsomal pellet was either washed in reaction buffer as described below or further purified by centrifugation on sucrose. Approximately 0.5 ml of microsomes resuspended in a minimal volume of lysis buffer with gentle dounce homogenization were loaded onto a 2.0 ml sucrose step gradient (1.0 ml each of 1.5 M sucrose and 1.2 M sucrose in lysis buffer) and centrifuged at 100,000 x g for 1 hr at 4°C. The microsomes at the 1.2 M/1.5 M interface were collected, washed twice in reaction buffer by centrifugation at 12,000 x g for 10 min, and resuspended in the same buffer (3 mg protein/ml). The high speed pellet (HSP) fraction was prepared from the 12,000 x g supernatant by centrifugation at 100,000 x g for 1 hr at 4°C. The pellet was washed once in reaction buffer (250 mM sorbitol, 20 mM Hepes, pH 6.8, 150 mM KOAc, 5 mM MgOAc) by centrifugation at 200,000 x g for 30 min at 4°C and resuspended in the same buffer with dounce homogenization (15 mg protein/ml).

Protein concentrations were determined in the presence of 1% SDS by the procedure of Lowry *et al.* (1951) using BSA as a standard. Samples were either used directly or frozen in liquid N_2 and stored at -80°C. No significant loss of activity was detected over 3 months of storage.

Microsome-Based Transport Reaction.

Transport reactions were carried out in two stages. In the first stage, 20 µg microsomes were mixed with 4 µl ^{35}S-prepro-α-factor in 50 µM GDP-mannose, 1 mM ATP, 40 mM creatine phosphate, 200 µg/ml creatine phosphokinase in a total volume of 12 µl reaction buffer. After incubation for 15 min at 10°C, the reaction mixture was diluted ten-fold with reaction buffer and microsomes were collected by centrifugation at 12,000 x g for 10 min. In the second stage, 10-20 µg washed, pro-α-factor-containing microsomes were mixed with 100 µg cytosol, 20 µg HSP, 50 µM GDP-mannose, 1 mM ATP, 40 mM creatine phosphate, 200 µg/ml creatine phosphokinase in a total volume of 50 µl reaction buffer and incubated for 90 min at 20°C. The amounts of cytosol and HSP added were

titrated for each preparation to give optimal transport efficiency. The reactions were terminated by addition of 50 µl 2% SDS, heated for 5 min at 95°C and precipitated with Con A-Sepharose or anti-α-1→6-Man serum and Protein A-Sepharose as described (Baker et al., 1988).

Results

Ypt1 Antibodies Block Transport.

Protein transport from the ER to the Golgi complex has been reconstituted using broken yeast spheroplasts (Baker et al., 1988). Transport is measured through the coupled addition within the Golgi complex of outer chain carbohydrate to core glycosylated ^{35}S-methionine labeled α-factor precursor originating from the ER. We took advantage of the observation that yeast microsomes are competent for transport (Ruohola et al., 1988) to develop a transport assay that shared the same basic features (ATP and temperature dependence, NEM and GTPγS sensitivity) as transport with broken spheroplasts but required ten fold less membrane protein (see MATERIALS AND METHODS).

Antibody inhibition studies were used to investigate the role of Ypt1p in the wild type transport reaction. Affinity purified Ypt1 and TrpE antibodies were prepared from antiserum raised against a Ypt1-TrpE fusion protein (Segev et al., 1988). The Ypt1 antibodies completely inhibited a wild type transport reaction (Figure 1A, closed squares) while the control TrpE antibodies had little effect (closed circles). Addition of purified Ypt1p made in bacteria, sufficient to saturate the antibody relieved the inhibition (Figure 1A, open squares). To rule out the possibility that inhibition was due to membrane aggregation by the divalent antibodies, the experiment was repeated with monovalent Fab fragments. Anti-Ypt1 Fab fragments inhibited the transport reaction (Figure 1B, open symbols). Again, the block was relieved by addition of excess bacterially-produced Ypt1p (Figure 1B, closed symbols).

In vivo studies have suggested that Ypt1p may function in both ER to Golgi and Golgi-Golgi transport (Segev et al., 1988; Schmidt et al., 1988). Core glycosylated invertase accumulated at the restrictive temperature in the *ypt1*-ts mutant whereas partially outer chain glycosylated invertase accumulated in the *ypt1-1* mutant. The anti-α1→6 linked mannose antibodies used to quantify the results of transport reactions do not react with glycosylated forms of the α-factor precursor migrating just above the core glycosylated ER form on SDS gels (Baker et al., 1988). To determine the effect of the Ypt1 antibody on the production of these partially outer chain glycosylated species, the radioactive α-factor precursor accumulated in an antibody inhibition experiment was displayed using SDS gel electrophoresis (Figure 1C). There was no significant accumulation of partially outer chain glycosylated species in the presence of the anti-Ypt1 Fab fragments (compare lane 3 to lane 1). Thus Ypt1p is involved in a transport partial reaction that precedes arrival in the first Golgi cisterna capable of outer chain glycosylation.

ypt1-1 Mutant Components Are Defective in Transport *in vitro*.

To investigate further the role of Ypt1p in transport *in vitro*, transport reactions were performed using components prepared from *ypt1-1* mutant cells . In the microsome based transport assay, the microsomes provide a source of translocation-competent ER but transport in addition requires both a cytosol and a high speed pellet (HSP) fraction (Table 1; Linda Wuestehube did this experiment). Immunoblotting analysis showed that the microsome, cytosol, and HSP fractions contribute 10%, 42%, and 48% of the total Ypt1p in a transport reaction. The experiments with mutant fractions contained HSP and microsomes from the same *ypt1-1 pep4* strain (These experiments was done by Nava Segev). Cytosolic fractions were prepared from an isogenic pair of Pep^+ strains differing only by the *ypt1-1* mutation. Transport was defective in reactions containing all ypt1-1 components (Figure 2, closed symbols). Cytosol prepared from the isogenic wild type strain restored transport (Figure 2, open symbols). Normal transport also occurred when

mutant cytosol and microsomes were incubated with a wild type HSP fraction (data not shown). Thus the efficiency of transport appears to reflect the amount of wild type Ypt1p rather than the origin of a particular fraction.

The *in vitro* Transport Reaction Requires Calcium and Ypt1p Independently.

The combination of the mutant and antibody studies argues strongly that Ypt1p plays a role in the *in vitro* transport reaction. As a connection has been proposed between Ypt1p and calcium flux (Schmitt et al., 1988), we wanted to determine whether Ypt1p functioned through calcium *in vitro*. A calcium requirement has been demonstrated in a mammalian ER to Golgi transport reaction (Beckers and Balch, 1989). Transport in semi-intact CHO cells was inhibited by 5 mM EGTA and restored when the free calcium concentration was adjusted to 0.1 μM. EGTA (5 mM) also inhibited the yeast ER-Golgi transport assay (Table 2, lane 1). Transport was only partially restored by calcium (Table 2, line 3). Manganese is required by the mannosyltransferases responsible for the outer chain carbohydrate elongation that is measured in the transport assay (Nakajima and Ballou, 1975) and EGTA has a high affinity ($K_d \approx 10^{-12}$ M) for manganese. Addition of manganese alone did not relieve inhibition by EGTA (Table 2, line 2). Transport was fully restored when an EGTA-treated reaction was supplemented with both cations to yield free concentrations of 120 nM calcium and 1.2 nM manganese (Table 2, line 4). Transport was optimal at about 100 nM Ca^{2+} (Figure 3), which is the estimated free intracellular calcium concentration in yeast (Hidetoshi Iida, personal communication). At higher concentrations of calcium protein transport was partially inhibited. It is likely that the free Ca^{2+} concentration in reaction buffers not supplemented with EGTA would exceed the inhibitory concentration in the transport reaction. Microsomes contain a potent Ca^{2+}-sequestering pump (A. Antebi and I, unpublished observations) which may explain why reactions proceed in the absence of EGTA.

It is possible that Ypt1p exerts its effect by modulating calcium flux *in vitro*. To investigate this possibility, anti-Ypt1 Fab fragments were added to transport reactions having free calcium concentrations ranging from 10^{-9} to 10^{-5} M. The anti-Ypt1 Fab fragments inhibited transport at all calcium concentrations tested (Figure 3, open symbols). Thus Ypt1p does not function solely by controlling calcium levels *in vitro*.

Inhibition by EGTA is reversible in the mammalian ER-Golgi reaction. This was also the case in the yeast transport reaction: transport resumed when calcium was added back to an EGTA treated reaction (Figure 4, column 7). This allowed us to order the requirements for calcium and Ypt1 *in vitro*. Standard transport reactions containing EGTA and manganese were either supplemented immediately with calcium (Figure 4, column 2-6) or pre-incubated for 60 minutes at 20°C and then supplemented with calcium. To compare the requirements of transport from the ER and transport from the calcium requiring intermediate, aliquots of both preincubated and fresh reactions were treated with either the ATP scavenger apyrase, the alkylating agent N-ethylmaleimide (NEM), anti-Ypt1 Fab, or the guanine nucleotide analogue GTPγS. As previously reported, all of the treatments blocked transport from the ER to the Golgi (Figure 4, columns 3-6). Transport from the calcium requiring intermediate was ATP dependent (column 11), cytosol dependent (M. Rexach, unpublished observations), and NEM sensitive (column 8). These results, coupled with the fact that the outer chain mannosyltransferases require manganese and not calcium as a cofactor, suggest that the calcium dependent step is a transport partial reaction rather than glycosylation. By analogy with results obtained in the mammalian transport reaction we suspect that the NEM sensitive factor is Sec18p, the yeast homologue of the mammalian NSF protein (Beckers et al., 1989; Wilson et al., 1989). The reactions that had been preincubated for 60 minutes in the absence of calcium no longer were inhibited by the anti-Ypt1 Fab fragments or by GTPγS (lanes 9, 10). The transport partial reaction(s) sensitive to the Ypt1 antibodies and GTPγS therefore either precedes the calcium requiring step or occurs on an independent pathway.

Discussion

We have demonstrated that Ypt1p is required for protein transport *in vitro*. ypt1-1 mutant components are defective in transport and Ypt1 antibodies inhibit transport in wild type extracts. These results support the recent proposal, based on analysis of the ypt1-1 mutant phenotype *in vivo*, that Ypt1p is involved in early stages of the yeast secretory pathway (2). The pleiotropic effects of ypt1 mutations *in vivo* left open the possibility that the transport defect was a secondary consequence of some perhaps unknown primary lesion. The biochemical data presented in this chapter argue that Ypt1p is directly involved in transport.

The yeast transport reaction requires about 100 nM free calcium. The overall calcium dependence of the reaction is similar to that of mammalian ER-Golgi transport, providing further evidence that the mechanisms underlying transport have been highly conserved through evolution. However, inhibition at greater than physiological calcium concentrations is less pronounced in the yeast reaction: 1 μM free calcium causes 75% inhibition of the mammalian reaction and only 20% inhibition of the yeast reaction. This is consistent with the proposal that the transient increase in free calcium is responsible for the inhibition of transport during mitosis in mammalian cells (Beckers and ıalch, 1989). As transport is not blocked during mitosis in yeast (Makarow, 1987), such a calcium "shut off" mechanism would not be expected in the yeast transport reaction.

Ypt1p functions independently of calcium in transport. Calcium cannot suppress inhibition of Ypt1p function in transport, and Ypt1p and calcium are required in different transport partial reactions. This does not exclude the possiblity that Ypt1p regulates calcium flux in addition to playing a direct role in transport.

Wild type Ypt1p in either the cytosol or HSP fractions can restore transport activity to a *ypt1-1* mutant reaction. One interpretation of this result is that the functional form of Ypt1p may cycle between the cytosol and a membrane target. Hence, complementation by the cytosolic fraction may require that the soluble Ypt1p be recruited to a functional binding site as proposed for Sec4p (Walworth et al., 1989). Alternatively, the soluble fraction may contain particles or small membrane fragments that are functionally equivalent to the form that sediments along with membranes. Preliminary sizing column analysis suggests that the active form of Ypt1p in soluble fractions may be associated with a large protein complex or a small vesicle (appendix 3).

Two other genes that encode small GTP-binding proteins have recently been implicated in early steps of protein transport in yeast. *SAR1* was identified as a multicopy suppressor of a *sec12* mutation (Aki Nakano, personal communication); mutants defective in a gene encoding ADP-ribosylation factor (*ARF1*) have a *ypt1*-like phenotype (T. Stearns, M. C. Willingham, D. Botstein, and R. A. Kahn, in preparation). It is possible that each of these GTP-binding proteins plays a role in a separate sub-reaction in early protein transport.

As discussed previously (Bourne, 1988; Baker et al., 1988), GTP binding proteins involved in transport could function in a manner similar to either the signal transducing G proteins or the elongation factors of protein synthesis. We would like briefly to elaborate on the analogy to protein synthesis. Protein synthesis and protein transport share the common requirements of unidirectionality, speed and accuracy. Peptide bond formation from aatRNAs has a ΔG of -7 kcal and thus will proceed unidirectionally in the absence of GTP and EfTu (Gavrilova et al., 1976). However, GTP binding proteins greatly increase the rate and accuracy of protein synthesis. The rate of polypeptide chain elongation is increased over 100 fold by EfTu and EfG (Gavrilova et al., 1981). By coupling GTP hydrolysis to a slow recycling step, a GTPase could similarly increase the rate of protein transport. EfTu increases the fidelity of protein synthesis through a "kinetic proofreading"

mechanism in which the aminoacyl tRNA-codon interaction is tested before and after GTP hydrolysis (Hopfield, 1974). GTP binding proteins could increase the accuracy of protein sorting through a similar proofreading mechanism. Thus, GTP binding proteins may guarantee the specificity of the interaction between secretory vesicles and acceptor components. These hypotheses might now be closer to being tested through the study of the role of Ypt1p in the *in vitro* transport reaction.

Table 1. Cytosol and High Speed Pellet Are Required for Transport

Addition	Anti-α1→6-Man Precipitable cpm (% Maximum)
Cytosol (100 μg) + HSP (20 μg)	100
Cytosol (100 μg)	8
HSP (20 μg)	8
Reaction buffer	7

In a two-stage reaction, pro-α-factor-containing microsomes were prepared as described in MATERIALS AND METHODS and then incubated with the additions indicated above in 50 μl reaction buffer containing GDP-mannose and an ATP-regenerating system. Reactions were terminated after 1.5 hr by addition of 2% SDS and reaction products were analyzed by immunoprecipitation followed by scintillation counting. A typical complete reaction yielded ~1000 cpm of α1→6-Man precipitable cpm which represented ~25% of the translocated pro-α-factor.

Table 2. Calcium Is Required for Transport

	Calcium		Manganese		α1-6 Man
	Free (nM)	Total (uM)	Free (nM)	Total (uM)	precipitable cpm
1.	<1	<5	<0.1	<5	90
2.	<1	<5	1.5	100	100
3.	120	500	<0.1	<5	360
4.	120	500	1.7	100	846

Standard transport reactions were supplemented with 5 mM EGTA and the indicated concentrations of calcium and manganese. The resultant free cation concentrations were estimated using a modification of a previously described computer program (see appendix 4). Addition of these concentrations of cations did not affect the pH of the incubations (data not shown). ConA precipitable pro-α-factor averaged 5500 cpm per point. A typical reaction without EGTA gave 950 cpm of transported pro-α-factor.

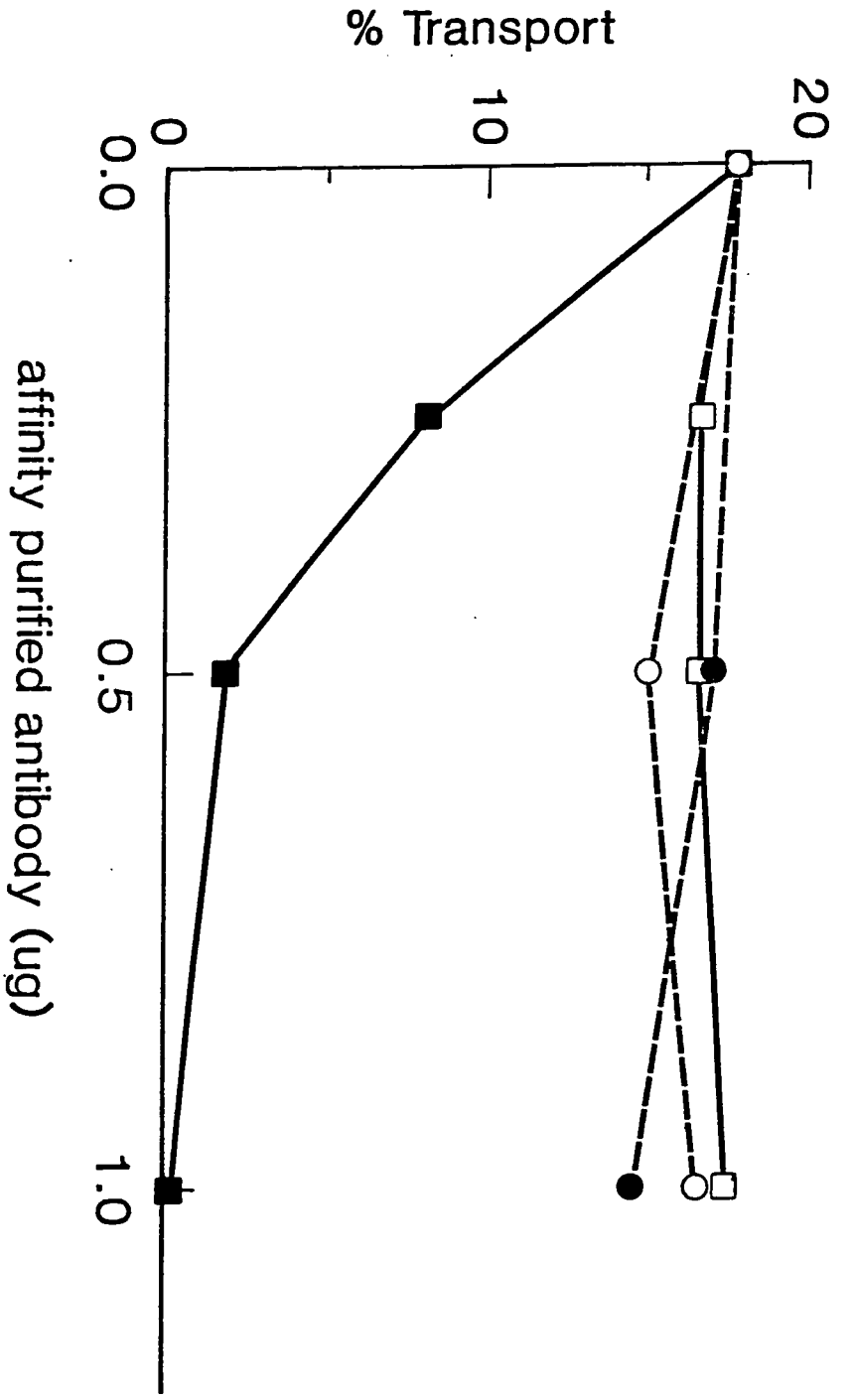
Figure 1. Anti-Ypt1 antibodies inhibit transport.

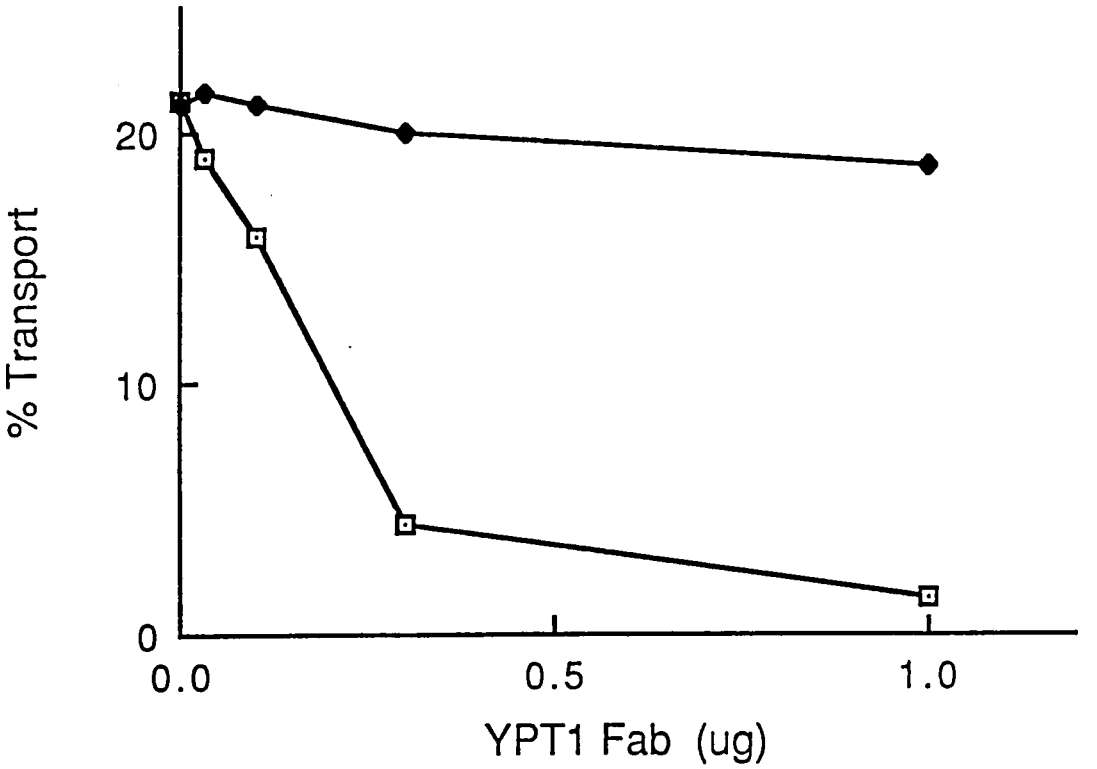
Standard transport reactions were performed as described in the MATERIALS AND METHODS with various additions:

(A) Increasing concentrations of affinity purified anti-TrpE (circles) or anti-Ypt1 antibodies (squares) were added to transport reactions containing (open symbols) or not containing (closed symbols) 0.75 μg purified Ypt1.

(B) Increasing concentrations of Fab fragments were added to transport reactions containing (closed symbols) or not containing (open symbols) 0.75 μg purified Ypt1.

(C) Standard transport reactions were supplemented with 1% SDS (reaction 1), 0.5 μg YPT1 Fab (reaction 3), or 0.5 μg YPT1Fab and 1 μg Ypt1p (reaction 4) as indicated. After 90 min at 20°C 1% SDS was added to reactions 2, 3 and 4. All reactions were then subjected to immunoprecipitation with anti-α-factor antibody, SDS gel electrophoresis and fluorography as described previously (7). Abbreviations: αf, α-factor precursor; cg, core glycosylated; ocg, outer chain glycosylated.


% Transport
0
10
20
0.0
0.5
1.0
affinity purified antibody (ug)


% Transport
20
10
0
0.0
0.5
1.0
YPT1 Fab (ug)

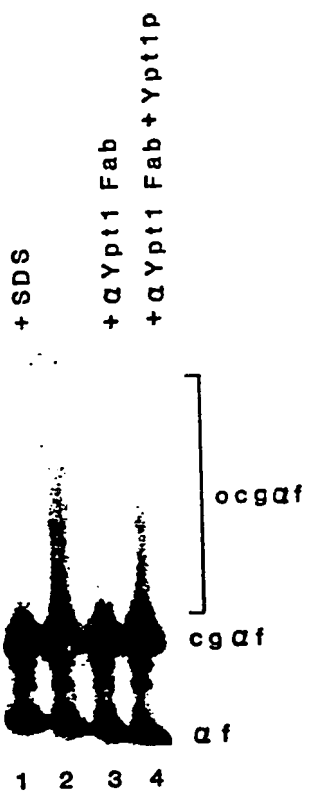

+ SDS
+ αYpt1 Fab
+ αYpt1 Fab + Ypt1p
ocgαf
cgαf
αf
1 2 3 4

Figure 2 *ypt1-1* mutant block in *in vitro* protein transport.

Microsomes and HSP (high speed pellet) fractions were prepared from a *ypt1-1* mutant strain (NSY12). Cytosols were prepared from wild-type (open symbols) (DBY1034) and ypt1-1 (closed symbols) (DBY1803) cells. *In vitro* reactions were performed as described in MATERIALS AND METHODS.

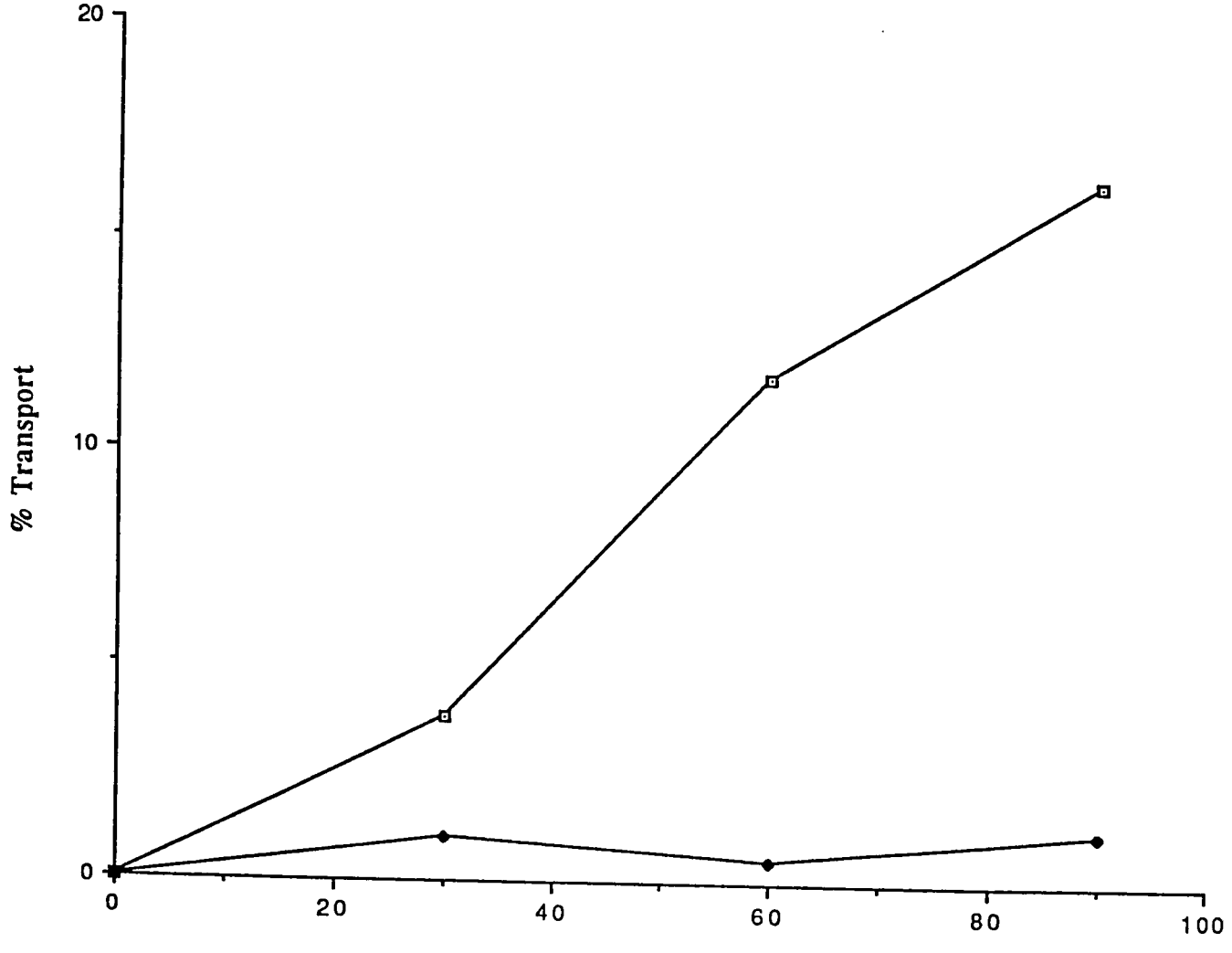

20
10
0
% Transport
0
20
40
60
80
100
minutes at 20°C

Figure 3. Calcium does not relieve inhibition by Ypt1 antibodies.

Standard transport reactions were performed in the presence of an EGTA/calcium/manganese buffer to yield the indicated free calcium concentration and 1-9 nM free manganese. The buffers were adjusted to pH 6.8 with KOH prior to addition to the reactions. Variation of manganese in this range did not affect transport (data not shown). The reactions were further supplemented with 0.5 μg anti-Ypt1 Fab fragments (closed symbols) or a buffer control (open symbols).

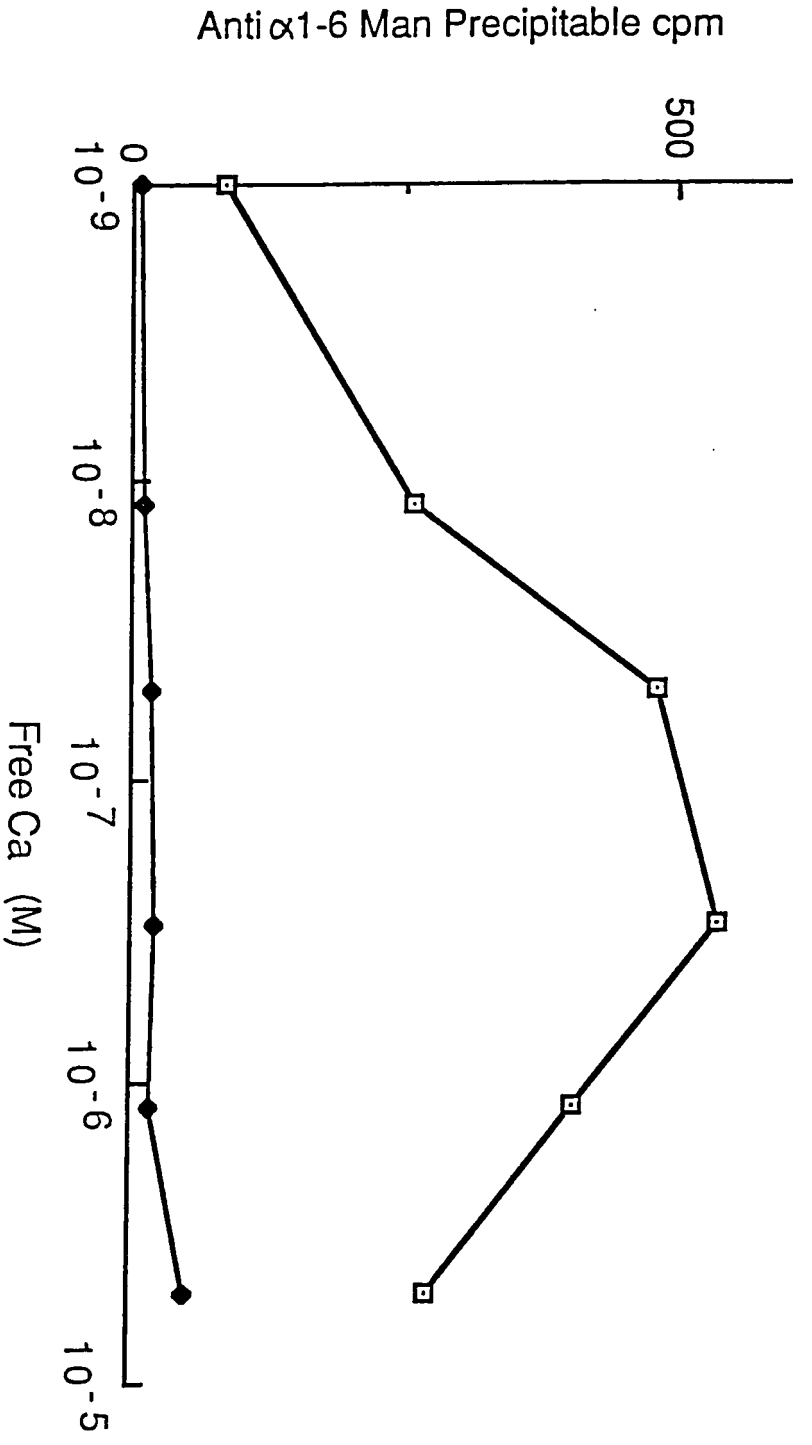
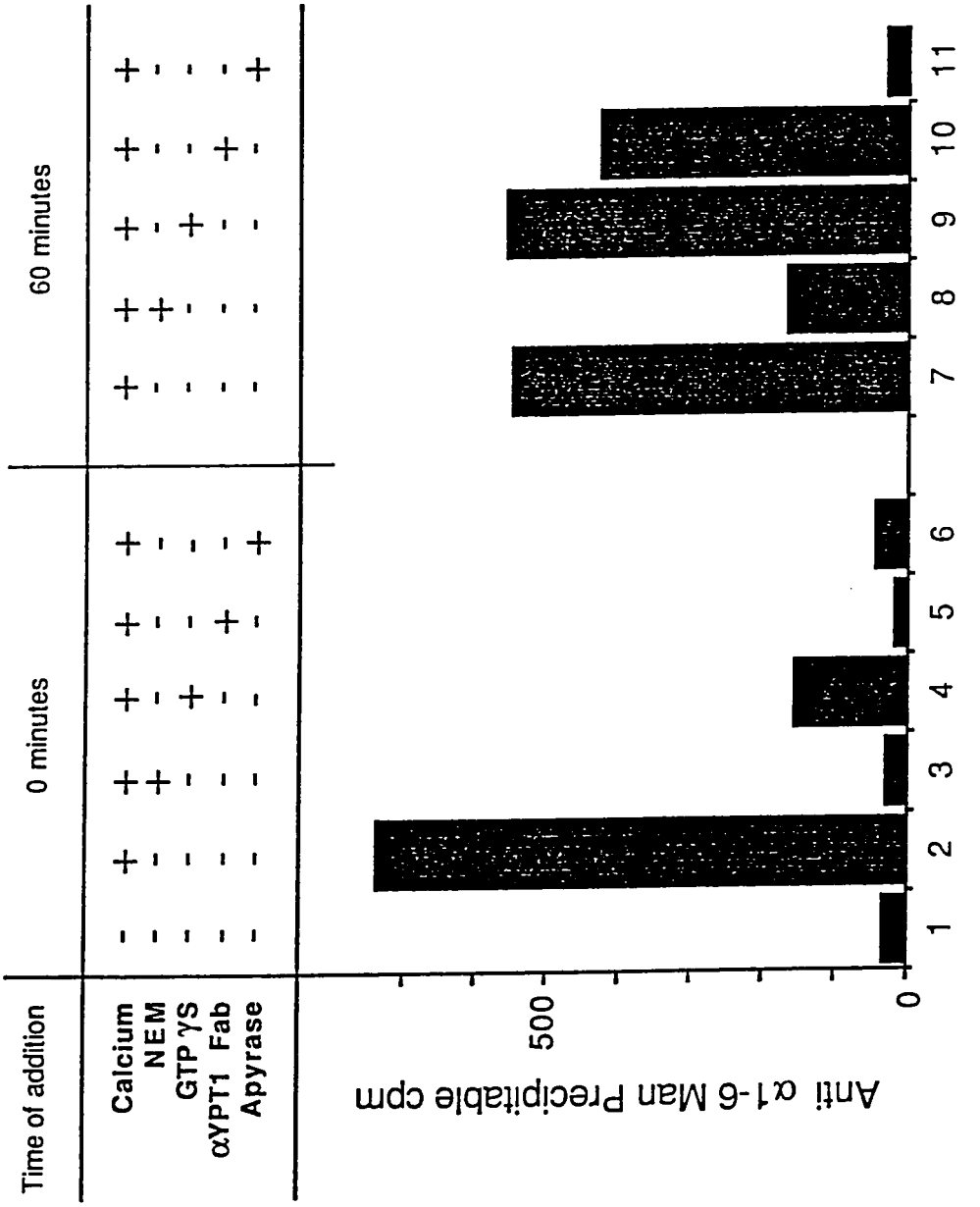

Anti α1-6 Man Precipitable cpm
500
0
10-9
10-8
10-7
10-6
10-5
Free Ca (M)

Figure 4. Anti-Ypt1 Fab fragments block transport before the calcium requiring step.

Standard transport reactions were performed in the presence of 5 mM EGTA and 100 μM manganese. After either 0 or 60 minutes of incubation at 20°C, the reactions were supplemented with NEM (to 10 mM), apyrase (2.5 u), GTPγS (100 μM) or anti-Ypt1 Fab fragments (0.5 μg) as indicated. After 10 min at 4°C, DTT (to 20 mM) was added to the NEM containing reaction. Calcium (500 μM) was then added and all reactions were incubated for an additional 90 minutes at 20°C. The final free calcium and manganese concentrations were 120 nM and 1.7 nM, respectively.


Time of addition
0 minutes
60 minutes
Calcium
NEM
GTP γS
αYPT1 Fab
Apyrase
Anti α1-6 Man Precipitable cpm
0
500
1
2
3
4
5
6
7
8
9
10
11

Appendix 1

Clathrin is not required for the receptor-mediated uptake of the mating pheromone α–factor

Summary

Clathrin heavy chain-deficient mutants (chc1) of Saccharomyces cerevisiae are viable but exhibit compromised growth rates. Receptor-mediated internalization of the mating pheromone α-factor occurred in chc1 cells at 36-50% wild-type levels. The results are consistent with the hypothesis that sequestration of G-protein coupled receptors occurs through a clathrin-independent pathway.

Introduction

Recent reports have presented evidence for internalization of the peptide pheromone α-factor by MATa yeast. α-Factor uptake bears the hallmarks of receptor-mediated endocytosis: uptake depends on time, temperature, energy and specific cell-surface receptors (Chvatchko et al., 1986; Jenness and Spatrick, 1986). Furthermore, peptide uptake is accompanied by a concomitant loss of cell-surface receptor activity, presumably due to receptor-ligand internalization (Jenness and Spatrick, 1986).

Sets of congenic clathrin-deficient (chc1) and wild-type (CHC1) strains have been generated by using single-step gene transplacement to disrupt CHC1 in haploid cells. Mutant strains grow 2 to 3 times more slowly than their wild-type counterparts (Payne and Schekman, 1985). In this study, we investigate the effect of CHC1 gene disruption on α-factor internalization.

Materials and Methods

^{35}S-Labelled α-factor was prepared and purified as described by Chvatchko et al. (1986). The specific activity of the purified peptide was approximately 1 Ci/mmole as determined by halo assay. Thin layer chromatography revealed two spots in accordance with results reported by Chvatchko et al. (1986).

For uptake determinations we applied a modification of the assay used by Chvatchko et al. (1986). Cells were grown overnight in YPD to an OD_{600} of about 1, then washed and

resuspended at 30 OD_{600}/ml in pH 5.5 YP plus 5.0% glucose, 5 mM p-tosyl-L-arginine methyl ester (TAME) and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). ^{35}S-labelled α-factor was added to a final concentration of 10^{-8} M and the cultures incubated with shaking at 24°C. At time intervals, 100 µl samples were removed and diluted into 2 mls of ice cold 50 mM sodium citrate, pH 2. After 20 minutes on ice, cells were collected on glass fiber filters (Whatman GFC) and washed with 20 ml of the citrate buffer. Filters were dried and counted in a liquid scintillation counter for 10 minutes with Beta-Max scintillant (West Chem). Cells (200 OD_{600}/ml) of strain GPY74-29B internalized 63% of 10^{-8} M α-factor in 75 minutes at 24°C. This value was taken as the percentage of ^{35}S-labelled peptide that was biologically active. In several experiments, replacement assays were conducted on 20 minute time point samples to determine the amount of biologically active α-factor remaining in the medium. Cells were removed by centrifugation and GPY74-29B cells added to 200 OD_{600}/ml and incubated at 24°C for 75 minutes. In each case (CHC1 MATa, chc1 MATa and chc1 Matα strains), the sum of the counts internalized by cells in the first 20 minute incubation and GPY74-29B cells in the replacement incubation corresponded to the amount of biologically active ^{35}S-labelled α-factor added at the outset.

For α-factor cell surface binding measurements, cells grown as described above were resuspended at various concentrations in pH 5.5 YP medium plus 10 mM potassium fluoride, 10 mM sodium azide, 5 mM TAME and 0.2 mM PMSF (inhibitor medium). ^{35}S-labelled α-factor was added to 10^{-8} M and the samples incubated with shaking at 24°C. After 25 minutes, samples were diluted into 2 ml inhibitor medium, collected onto glass fiber filters and washed 3 times with 2 mls inhibitor medium. Filters were dried and counted as above.

Results

A modified version of the assay described by Chvatchko et al. (1986) was employed to measure uptake of radiolabelled α-factor by wild-type and clathrin-deficient cells. Purified ^{35}S-labelled α-factor was added to chc1 sst1 MATa or CHC1 sst1 MATa cells (10^{-8} M α-factor, 3 x 10^8 cells/ml). The sst1 mutation eliminates a secreted protease which degrades α-factor (Ciejek and Thorner, 1979). After incubation at 24°C, cells were collected and washed with pH 2 buffer. pH 2 treatment strips α-factor bound to receptors at the cell surface leaving only internalized peptide associated with cells (Chvatchko et al., 1986; my unpublished results). In our first experiments, despite the sst1 mutation, exogenous α-factor was rapidly inactivated by chc1, but not CHC1, cells. Viable staining with methylene blue indicated that about 5% of the mutant cells were inviable whereas less than 0.1% of the wild-type cells were dead. It seemed possible that α-factor inactivation resulted from degradation by proteases released by inviable chc1 cells. To circumvent this problem, genetic and biochemical strategies were applied. First, prb1 (Zubenko et al., 1979) and pep4 (Hemmings, et al., 1981) mutant alleles were introduced into chc1 and CHC1 strains to lower the level of endogenous vacuolar proteolytic activity. Second, protease inhibitors p-tosyl-L-arginine methyl ester (TAME) (Ciejek, and Thorner, 1979) and phenyl methyl sulfonyl fluoride (PMSF) were added to the incubations. Under these conditions, both chc1 and CHC1 strains internalized α-factor (Figure 1A). The clathrin-deficient cells accumulated α-factor at nearly half the rate of wild-type cells. Figure 1B illustrates a compilation of results from several experiments. In four experiments, after 20 minutes at 24°C, uptake by chc1 strains ranged from 36-50% of wild-type levels (with "no energy" background subtracted). Internalization was temperature (not shown) and energy dependent (compare first two bars to third and fourth bars in Figure 1B). Finally, uptake of α-factor by chc1 cells was receptor-mediated since MATα chc1 cells which do not express α-factor receptor failed to take up pheromone (Figure 1B, fifth bar).

Reduced uptake of α-factor by chc1 strains did not result from degradation of exogenous pheromones or decreased binding capacities. Degradation was examined using a cell

replacement assay. An incubation of chc1 cells (either MATa or MATα) with labelled α-factor at 24°C was terminated after 20 minutes by sedimenting the cells. The supernatant fraction was then incubated with fresh CHC1 cells for 75 minutes at 24°C. The amount of radiolabel internalized during the two incubations equalled the amount of biologically active α-factor added initially (see Materials and Methods). Thus, during a 20 mintue incubation with mutant cells, no significant degradation of α-factor was observed. This experiment also indicated that the absence of α-factor internalization by chc1 MATa cells was not due to competition from unlabelled α-factor secreted by cells during the incubation. Binding of α-factor was measured at 24°C by incubating labelled pheromone with varying concentrations of cells in the presence of energy inhibitors to prevent internalization (Jenness et al., 1983; also see Materials and Methods). Bound α-factor was determined following a pH 5.5 wash. Figure 1C demonstrates that binding to chc1 and CHC1 cells was identical.

In the assay described by Chvatchko et al. (1986) internalized α-factor was rapidly degraded. We were unable to detect degradation of internal pheromone in either mutant or wild-type cells. This probably reflects the lowered proteolytic activity due to the prb1 and pep4 mutations. Riezman and his colleagues have made a similar observation using pep4 strains (personal communication).

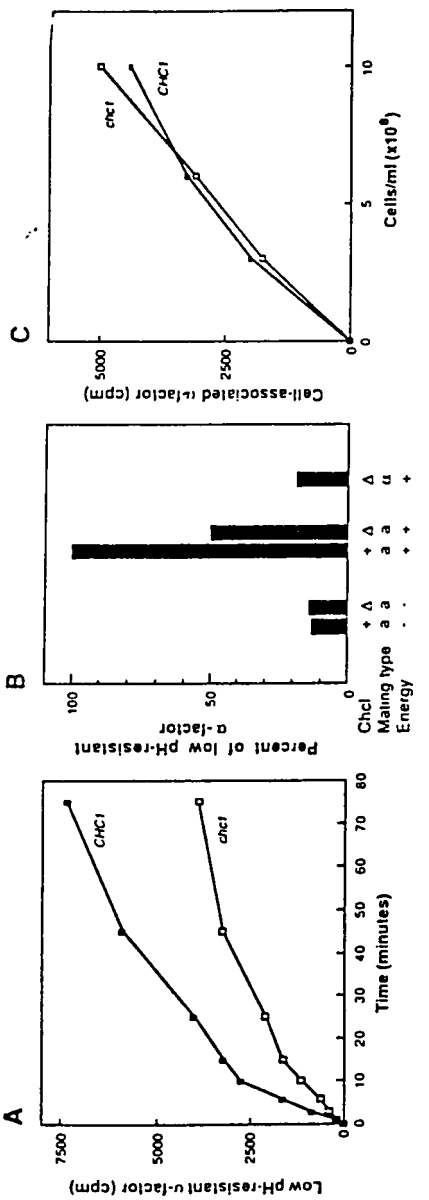
Discussion

During receptor mediated endocytosis, many receptors cluster in clathrin coated pits which subsequently pinch off to form clathrin coated vesicles (Goldstein et al, 1985). The cage forming properties of isolated clathrin triskelions have suggested that clathrin polymerization may drive the formation of endocytic vesicles. Our finding that receptor mediated uptake of α-factor occurs in cells containing no clathrin may appear to conflict with this idea. However, the α-factor receptor belongs to a class of receptors which has not been associated with clathrin coated pits or coated vesicles.

While the ligand of the α-factor receptor resembles those of other growth factor receptors, the structure of the receptor and the signalling pathway it impinges upon are more reminiscent of the β-adrenergic receptor. Most growth factor receptors have one or two membrane spanning domains and are thought to function through cytoplasmic kinase domains (Yarden and Ullrich, 1988). The α-factor receptor, in contrast, has seven predicted membrane spanning domains (Nakayama et al, 1985) and is coupled to a G protein (Dietzel and Kurjan, 1987). Clathrin has been implicated in the receptor mediated endocytosis of the growth factor receptors, such as the epidermal growth factor, with single membrane spanning domains (Goldstein et al, 1988). However, clathrin has not been associated with the sequestration of receptors, such as the β-adrenergic receptor, which have seven membrane spanning domains and are coupled to G proteins (Sibley et al, 1987). Our results are consistent with the hypothesis that the disappearance of this class of receptors from the cell surface occurs through a clathrin independent pathway.

Figure 1. Uptake and Binding of α-Factor by MATa chc1 and MATa CHC1 Strains

A) chc1 cells internalize α-factor. MATa CHC1 strain GPY74-29B (closed squares) and MATa chc1 strain GPY79.1 (open squares) were suspended at 3 x 10^8 cells/ml in YP plus 50% glucose, 5 mM TAME and 0.2 mM PMSF. α-Factor was added to 10^{-8} M and cells incubated at 24°C. At the designated times, cells were harvested and assayed for internalized α-factor by washing with pH2 buffer as described in the Materials and Methods. B) α-Factor uptake is energy and receptor-dependent. α-Factor was measured after 20 minutes at 24°C as described in A. Samples without energy contained 10 mM sodium azide and 10 mM sodium fluoride in place of glucose. The bars indicate average values obtained from multiple experiments using MATa CHC1 strain GPY74-29B, chc1-Δ10 derivatives 79.1 and 79.2 and chc1-Δ10 MATα strain GPY68. In each experiment the amount of α-factor internalized by the MATa CHC1 strain was taken as 100%. C) α-factor binding. GPY74-29B (CHC1) and GPY79.1 (chc1-Δ10) were incubated with 10^{-8} M α-factor in YP medium plus 10 mM potassium fluoride, 10 mM sodium azide, 5 mM TAME and 0.2 mM PMSF for 25 minutes at 24°C. Bound α-factor was measured by washing with the incubation medium as described in the Materials and Methods.


A
Low pH-resistant u-factor (cpm)
7500
5000
2500
0
0
10
20
30
40
50
60
70
80
Time (minutes)
CHC1
chc1
B
Percent of low pH-resistant
a-factor
100
50
0
Chcl
Mating type
Energy
+ Δ
a a
- -
+ Δ
a a
+ +
Δ
α
+
C
Cell-associated α-factor (cpm)
5000
2500
0
0
5
10
Cells/ml (x10⁶)
chc1
CHC1

Appendix 2

Freeze-thaw lysis

Introduction

Yeast spheroplast ghosts which support efficient protein translocation and intercomparmental transport reactions can be prepared by controlled freeze-thawing. The principal advantages of freeze-thaw lysis are high yield and convenience: spheroplasts can be prepared in large quantities and then frozen in aliquots which can be thawed when needed. Further, unlike more vigorous homogenization procedures, the method preserves the ER in an unfragmented state which can be readily separated from transport vesicles and other smaller particles by differential centrifugation. Freeze thaw lysis can be used to prepare minimally perturbed, highly concentrated cell extracts without tight constraints on buffer composition and thus may be well suited for reconstitution and investigation of other complex cell biological phenomena including signal transduction and cell cycle control.

A brief review of the stresses placed on cells during freeze- thawing is useful before a detailed description of the lysis procedure. The extent of lysis is determined largely by the rates of freezing and thawing (Grout and Morris, 1987). The principal source of damage during freeze-thawing is intracellular ice formation. When a cell suspension is cooled, the surrounding medium (having a larger volume and hence an increased probability of ice crystal nucleation) begins to freeze first. This creates an osmotic stress on the cells and water flows out across the plasma membrane. When the temperature is lowered sufficiently, any remaining intracellular water freezes. If the rate of freezing is sufficiently slow, most of the intracellular water is lost by dehydration and little intracellular ice forms. During rapid freezing however, low temperatures are reached before significant dehydration has occurred and internal water freezes. Slow freezing leads to less intracellular ice formation than rapid freezing and hence is less stressful. Conversely, rapid thawing, which allows less time for potentially damaging ice recrystallization, is less

stressful than slow thawing. By adjusting the rates of freezing and thawing the desired degree of lysis can be achieved.

Method

Preparation of actively metabolizing spheroplasts

Yeast cultures are grown in YPD medium (1% Bacto-Yeast extract (Difco), 2% Bacto peptone, and 5% glucose) to 2-4 OD_{600} U/ml (1 OD_{600} U is approximately 10^7 cells). Wild type strains are grown at 30°C, temperature sensitive (ts) strains at 24°C. Cells are harvested by centrifugation (1000 x g, 5 min, 24°C) and resuspended at 50 OD_{600} U/ml in 10 mM DTT, 100 mM Tris HCl pH 9.4. After 5 minutes at 24°C, cells are harvested by centrifugation and resuspended at 50 OD_{600} U/ml in 0.7 M sorbitol, 0.75 x YP (1% Bacto yeast extract, 2% Bacto peptone), 0.5% glucose, 10 mM Tris HCl pH 7.5 (25 ml 2.8 M soribtol, 75 ml YP, 1 ml 50% glucose and 1 ml 1 M Tris HCl, pH 7.5, per 100 ml of medium) and cell walls are digested with 20 U lyticase per OD_{600} U cells (Scott and Schekman, 1980). Zymolyase (Miles) can probably be used in place of the lyticase. The cell suspension is incubated at 30°C (24°C for ts strains) until the OD_{600} of a 1:100 dilution in H_20 drops to less than 10% of the initial value. The incubation to form spheroplasts should not be longer than 20 minutes; we have had poor results with longer incubations. The rate of synthesis of at least one protein, invertase, drops dramatically under spheroplasting conditions (Manfred Schleyer, unpublished observations). To allow protein synthesis to resume, spheroplasts are harvested by centrifugation, resuspended at 6 OD_{600} U/ml in 0.7 M sorbitol, 0.75 x YP, 1% glucose and incubated with gentle shaking for 20 min at 30°C (24°C for ts strains).

Freezing conditions

Two variables are important: the freezing rate and the buffer composition. In the absence of sophisticated equipment to control the cooling rate (Grout and Morris, 1987),

we have used simple but reproducible methods. Rapid, intermediate, and slow rates of freezing are achieved by direct immersion in liquid N_2, suspension over liquid N_2 vapor, and placement in an insulated box in a -85°C freezer, respectively. The lysis buffer contains 400 mM sorbitol, 150 mM KOAc, 2 mM MgOAc, 0.5 mM EGTA, and 20 mM Hepes, pH 6.8. The Mg^{2+} is required to stabilize nuclei, the pH and salt concentrations approximate physiological conditions, and the EGTA chelates any Ca^{2+} released during lysis. Higher sorbitol concentrations reduce the lysis efficiency and lower concentrations lead to osmotic lysis.

Biosynthetically active spheroplasts are harvested by centrifugation (1000 x g, 5 min, 4°C), washed and resuspended at 300 OD_{600} U/ml in lysis buffer at 4°C. Aliquots of the spheroplast suspension are transferred to 1.5 ml Eppendorf microcentrifuge tubes and frozen in liquid N_2 vapor (see below). Smaller aliquots freeze more rapidly than large aliquots and the degree of lysis can be controlled through the aliquot size. Aliquots of 200 µl are used to prepare membranes for the ER-Golgi transport assay; smaller aliquots (50-100 µl) give more efficient lysis but lower transport efficiency.

The following procedure is used to freeze spheroplasts in liquid N_2 vapor: A styrofoam ice bucket is filled with liquid N_2 to a height of about 6 cm. A cardboard freezer box divider is suspended at a level even with the top of the bucket with wire attached to each corner, approximately 10 cm above the liquid N_2. Eppendorf tubes containing 50-200 µl portions of spheroplast suspension are placed in the cardboard rack and the ice bucket is covered and then sealed with tape. After 40 minutes the tubes are transferred to a -85°C freezer. Membranes have been stored for two months at -85°C without loss of activity. Tubes are thawed by immersion in a 25°C water bath with shaking and then placed on ice.

For some applications it may be useful to freeze spheroplasts slowly and thereby preserve them essentially intact (see below). Slow freezing provides a means to store large quantities of radiolabeled or otherwise specially prepared spheroplasts. For example, reconstitution of transport to the yeast vacuole has been acheived using metabolically

labeled, frozen spheroplasts that are broken by osmotic lysis after thawing (Tom Vida, personal communication).

Characterization of freeze-thaw lysates

The extent of lysis can be easily monitored by phase contrast microscopy. Intact spheroplasts have a characteristic bright halo resulting from the sharp difference in refractive index between spheroplast and surrounding buffer (Figure 1a). Freeze-thawing produces ghosts which can be differentiated from intact spheroplasts by the absence of the halo, presumably due to loss of cytoplasmic contents (Figure 1c, e). Ghosts with similar appearance are produced when spheroplasts are diluted into low osmotic support buffers.

The integrity of the nuclei and the permeability of the plasma membrane can be quickly assessed using the DNA binding fluorescent dye 4,6-diamidino-2-phenylindole (DAPI) (Sigma). Addition of 20 μg/ml DAPI to unfixed, intact spheroplasts gives rise to the faint staining pattern seen in Figure 1b. Freeze-thaw ghosts stained under identical conditions produce a much stronger signal (Figure 1d; the exposure time in 1b is twice that of 1d). A field containing ghosts and an intact spheroplast is shown in Figure 1f and g for direct comparison of DAPI staining intensity. The increase in staining intensity is most likely due to permeabilization of the plasma membrane and not the nuclear envelope: invertase accumulated within the nuclear envelope/ER in a *sec18* mutant is not released during feeze-thawing and the nuclear envelope appears intact by electron microscopy (see below)

The degree of permeabilization of the plasma membrane and the ER can be assessed by monitoring the release of the cytoplasmic and secreted forms of invertase. In a *sec18* mutant at the non-permissive temperature, core glycosylated invertase accumulates in the ER (Esmon *et al.*, 1981). *sec18* Spheroplasts were prepared as described above and shifted to 0.7 M sorbitol, 0.75 x YP, 0.1% glucose for 20 min at 24^{o}C to derepress invertase synthesis and then to 37^{o}C to accumulate core-glycosylated invertase in the ER. The spheroplasts were then suspended in lysis buffer and frozen at different rates. After

thawing, lysates were centrifuged for ten seconds at 12,000 x g in a Fisher microcentrifuge and the pellet and supernatant fractions analyzed by native gel electrophoresis followed by an invertase activity stain. Freezing over liquid N_2 vapor caused release of cytosolic but little core-glycosylated invertase from the sedimentable to the supernatant fractions (Figure 2, lanes 2p, 2s). Both forms of invertase were sedimentable in samples containing spheroplasts prior to freezing (lanes 1p, 1s) or spheroplasts frozen slowly in an insulated freezer box (lanes 3p, 3s). Approximately 40% of the core glycosylated invertase was released into the supernatant following rapid freezing in liquid N_2 (data not shown). Thus slow freezing rates leave spheroplasts intact, intermediate freezing rates permeabilize the plasma membrane but not the ER, and rapid freezing rates permeabilize both the plasma membrane and the ER. Spheroplasts remain viable when frozen sufficiently slowly: invertase accumulated in the ER was quantitatively secreted when spheroplasts frozen in an insulated box were thawed and incubated in YPD plus 0.7 M sorbitol (data not shown). The extent of lysis of spheroplasts and vacuoles during preparation of wild-type membranes for the ER-Golgi assay was monitored by immunologic detection of the cytosolic enzyme phosphoglycerate kinase (PGK) (serum provided by J. Thorner, this Dept.) and the vacuolar protease carboxypeptidase Y (CPY) (Stevens *et al.*, 1982). Immunoblotting was used to show that approximately 50% of the PGK remained soluble after 10 second of centrifugation in a microcentrifuge while greater than 90% of the CPY sedimented. Vacuoles were sensitive to rapid freezing: greater than 60% of the CPY remained soluble when spheroplasts were lysed by direct immersion in liquid N_2 (data not shown).

Figure 1. Morphology of broken spheroplasts: light microscopy.

Spheroplasts before (A, B) and after (C, D, E, F, G) freezing in liquid N_2 vapor were suspended in lysis batter containing 20 μg/ml DAPI and examined using phase contrast (A, C, E, F) or fluorescence (B, D, G) microscopy. The exposure time was six seconds in B and three seconds in D and G. The magnification in A, B, C, D, F, G was 400x and in E, 630x.

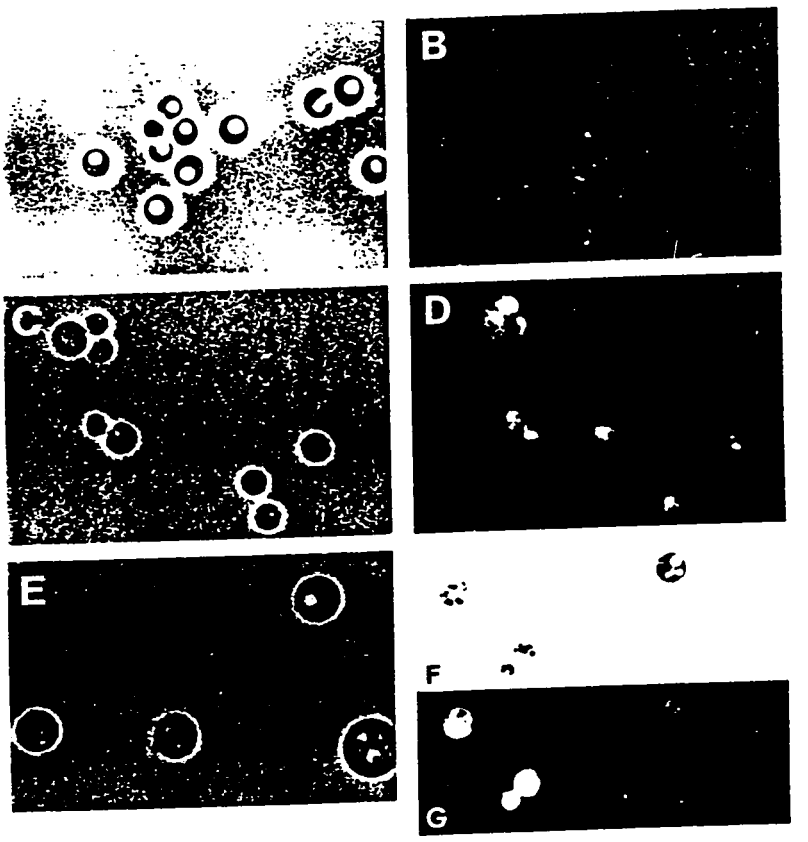
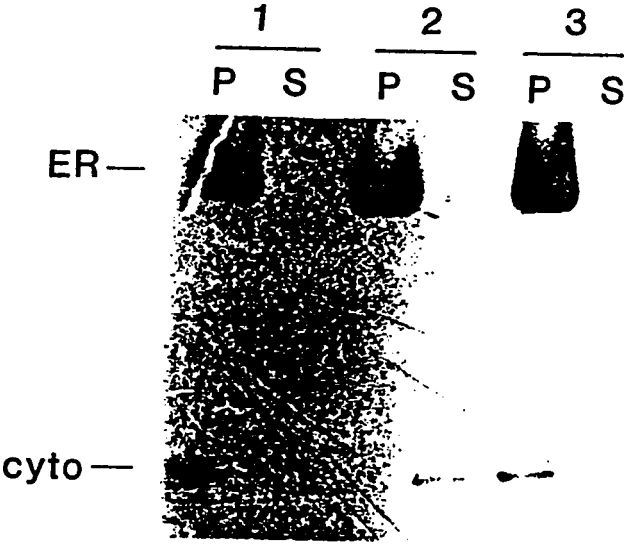

B
C
D
E
F
G

Figure 2. Invertase fractionation.

Pellet and supernatant frctions were prepared from *sec18* spheroplasts prior to freezing (1), after freezing in liquid N_2 vapor (2), or after freezing in an insulated box (3), as described in the text. The fractions were adjusted to 0.2% Tx-100, 5 mM Mes pH 6.5 and heated for 15 min at 50°C to dissociate invertase oligomers. Glycerol and bromphenol blue were then added to final concentrations of 10% and 0.1%, and portions were subjected to native gel electrophoresis (5 OD/U equivalents of extract per lane) through a 7% polyacrylamide gel buffered with 0.1 M Tris-HCl pH 7.5. The gel was run in 120 mM Tris Borate pH 7.5 for 3 hours at 10m Amp. Invertase activity was detected following electrophoresis by incubating the gel first in 0.1 M sucrose, 0.1 M NaOAc pH 5.1 for 30 min at 30°C and then in 0.1% 2,3,5-triphenyltetrazolium chloride, 0.5 M NaOH for 4 min at 90°C. P, pellet fraction; S, supernatant fraction. ER, core-glycosylated invertase; cyto, cytoplasmic invertase.


1
2
3
P S P S P S
ER
cyto

Appendix 3

A large Ypt1p containing particle is required for protein transport in vitro

Summary

An immunodepletion approach was used to examine the role of Ypt1p in wild type transport reactions. In reactions containing low concentrations of microsomes or gently-lysed cells, transport required a cytosol fraction and a high salt extract of membranes. Ypt1p was depleted from cytosol and salt extract by treatment with Ypt1 antibodies coupled to protein A Sepharose beads. Immunodepletion of both fractions blocked transport. Supplementation with either the salt extract or a high speed pellet (HSP) fraction restored transport activity to immunodepleted reactions. The rescuing activity in both fractions comigrated with Ypt1p on gel filtration columns. The size of the Ypt1 associated activity suggests either a small membrane vesicle or a large protein complex. In an independent set of experiments, a putative transport intermediate was found to accumulate in Ypt1 defective reactions. Comparison of the fractionation behavior of the Ypt1p associated activity in the salt extract, one of the two Ypt1p associated particles in the HSP, and the Ypt1p requiring transport intermediate suggests that they may be related objects.

Materials and Methods

Unless otherwise indicated, all materials and procedures were exactly as described in chapter 3.

Preparation of salt extract

Washed spheroplasts prepared from 20,000 OD_{600} U of cells were suspended in 200ml of 20mM Hepes pH 6.8, 2mM MgOAc, 1mM DTT, 0.5mM PMSF at 4°C and centrifuged for 15 min at 100,000xg. The pellet was resuspended in 50ml of 20% saturated $(NH_4)_2SO_4$, incubated for 20min at 4°C, and centrifuged for 15min at 100,000xg. An equal volume of 80% saturated $(NH_4)_2SO_4$ containing 1mM DTT was added slowly to the supernatant while stirring. After an additional 5min at 4°C, the salt treated supernatant was

centrifuged for 10min at 100,000xg. The resulting pellet was resuspended in 10ml of reaction buffer containing 1mM DTT and dialyzed against the same buffer. Insoluble material was then removed by centrifugation for 15min at 100,000xg and the clarified salt extract was frozen in liquid N_2 and stored at -85 C.

For convenience, microsome and salt wash fractions were often prepared from frozen spheroplasts stored at -85 C. For freezing, spheroplasts prepared from 50000 OD_{600} U of cells were collected by centrifugation, resuspended in 60 ml 0.7M sorbitol and centrifuged through Ficoll cushions. The washed spheroplasts were resuspended in 0.7M sorbitol, aliquoted to 10ml plastic tubes and again collected by centrifugation. The pellets were frozen in an insulated freezer box at -85°C

Immunodepletion

Antibody coated beads were prepared by incubating 1 ml of packed protein A Sepharose beads with 2 ml of anti Ypt1 or preimmune serum and 1.5ml of PBS for 10 hours at 4 C with mixing. The beads were then washed 4x by sedimentation and resuspension in 7ml 0.2M triethanolamine pH 8.2. For crosslinking, the beads were resuspended in 10ml 10mM dimethyl pimelimidate, 0.2M triethanolamine pH 8.2, incubated for 40min at room temperature, and washed 2x with 10mM ethanolamine pH 8.2 and 2x with PBS.

For immunodepletion, 4 ml of cytosol or salt extract were mixed with 1 ml of the crosslinked beads and incubated with mixing for 60 min at 4 C. Small aliquots of the depleted fractions were then frozen in liquid nitrogen. The beads could be reused at least three times.

Results

Cytosol and a salt extract fraction support transport

Transport with microsomes or low concentration of gently lysed cells requires a high speed pellet fraction (HSP) in addition to cytosol (chapter 3, table 1). We explored salt extraction of membranes as a means to solubilize the factors sedimenting in the HSP. Extraction of membranes with 1M KCL or 1M KOAc followed by salt exchange, concentration with an Amicon 10 concentrator, and clarification at 100,000x g yielded a salt extract which restored transport activity to reactions containing only cytosol. The slow concentration step could be avoided by extracting membranes with 1M $(NH_4)_2SO_4$ and then precipitating the activity by addition of $(NH_4)_2SO_4$ to 50% saturation. The $(NH_4)_2SO_4$ extract and cytosol supported transport in a strongly synergistic fashion (table 1).

Immunodepletion of Ypt1p blocks transport.

The existing ypt1 mutations are not well suited for detailed in vitro analysis. In vivo, the ypt1-1 mutation is constitutively defective in transport (Segev et al., 1988) and the ypt1-ts mutant protein can have dominant lethal effects (Schmitt et al., 1988). Previous experience with the sec mutants suggested that defects observed in extracts prepared from cells blocked in transport in vivo may be indirect. Perhaps because of such pleiotropic defects, efforts to establish a complementation assay with ypt1-1 mutant extracts met with little success. A dominant inhibitor, probably the mutant protein, prevented rescue of ypt1-ts mutant extracts. A method for studying the role of Ypt1p in wild type extracts was therefore desirable.

We explored an immunodepletion approach. Reaction conditions were found in which less than 15% of the total Ypt1p was supplied by the microsome fraction. The Ypt1p in the salt extract and cytosol fractions was then depleted by treating the fractions with anti Ypt1 antibodies or control pre immune antibodies crosslinked to protein A Sepharose beads. Treatment with the anti Ypt1 beads removed over 75% of the Ypt1p from the salt extract (figure 1A, compare lanes 1 and 2) and cytosol (figure 1A, compare lanes 3 and 4)

fractions. Immunodepleted fractions were contaminated with less than .01ug of IgG (0.5ug of Ypt1 antibodies are required for half maximal inhibition of an in vitro reaction). The synergistic dependence of transport on the salt extract and cytosol was not affected by treatment with the preimmune coated beads (figure 1B, columns 1-3). Treatment of either salt extract or cytosol with the anti Ypt1 beads also had little effect on transport (column 4). However, treatment of both fractions with the anti Ypt1 beads abolished transport (column 6). Thus, transport absolutely requires a Ypt1p-associated activity that can be supplied in either fraction.

Supplementation of immunodepleted reactions with untreated fractions provided an assay for the characterization of the Ypt1p-associated activity (table 2). Cytosol, salt extract and HSP fractions differed in their ability to restore transport to immunodepleted reactions; full restoration required 80ug of cytosol, 10ug of salt extract or 3ug of HSP. Differential centrifugation of the salt extract showed that the rescuing activity is associated with a large (10-100s) particle (table 2). Purified recombinant Ypt1p had no detectable rescuing activity. As antibody inhibition is reversed by addition of recombinant Ypt1p, this result ruled out the possibility that the transport block in immunodepleted reactions was due to trace amounts of Ypt1 antibodies.

The rescuing activity in the salt extract and in the HSP was analyzed by gel filtration chromatography. The activity in the salt extract comigrated with the Ypt1 antigen on a Sephacryl S500 column, eluting in the included volume before the major peak of protein (figure 2; note peaks of antigen and activity in fractions 15 and 16). Decreasing amounts of activity were recovered from chromatography on resins (S300, S500, S1000) of increasing pore size. This may result from separation of two large particles or an instability on gel filtration columns similar to that reported for clathrin coated vesicles (Privat et al., 1987). Fractionation of the HSP by Sephacryl S1000 chromatography or by velocity gradient sedimentation separated two Ypt1p containing particles. The larger particle migrated in the void volume of the S1000 column and sedimented with an S value of greater than 350.

The smaller particle roughly comigrated with the Ypt1p containing particle in the salt extract on the S1000 column and sedimented with an S value of 80-140S. The heterogeneity on the S1000 column and on the velocity gradient may be due to aggregation of the smaller particles (vesicles?) in the gelatinous HSP. Release of clathrin coated vesicles from a similar HSP fraction requires treatment with RNase (Mueller and Branton, 1984). Intra Golgi transport vesicles have also been found to aggregate in HSP fractions (Vivek Malholtra, personal communication).

A putative transport intermediate accumulates in Ypt1 defective incubations

An assay developed by Michael Rexach was used to investigate the role of Ypt1p in the production of a slowly sedimenting putative transport intermediate. In the experiments described in table 3, the ypt1-1 mutation and anti Ypt1 Fab fragments blocked ER-Golgi transport as reported previously (see chapter 3). However, the appearance of core glycosylated α factor in a slowly sedimenting compartment was not inhibited in either case. Thus, Ypt1p is required at a stage following the formation of the intermediate, perhaps vesicle targeting or fusion.

Discussion

An immunodepletion approach was used to investigate the function of Ypt1p in wild type transport reactions. Depletion of Ypt1p from cytosol and salt wash fractions abolished transport. Supplementation with salt extract or HSP restored transport activity to immunodepleted reactions. The rescuing activity in both fractions was associated with large Ypt1p containing particles.

What are the large Ypt1p associated particles in the HSP and the salt extract? Similar salt extraction procedures have been used to release transport vesicles and large protein complexes such as ribosomes from more rapidly sedimenting membrane fractions. As Ypt1p is 50% identical to the secretory vesicle-associated Sec4p, the Ypt1 associated

activity could well be either a vesicle or a vesicle component. The HSP fraction appears to contain two Ypt1p associated particles, one of of >350S, the other of approximately the same size as the particle in the salt extract. The smaller particle could be a transport vesicle, while the larger particle could be Golgi vesicles or an aggregate of the smaller particles. Both clathrin coated vesicles and intra Golgi transport vesicles have been found to aggregate in similar high speed pellet fractions. The activity in the salt extract may be similar to the smaller particle in the HSP fraction. There may be two pools of transport vesicles within cells: vesicles peripherally associated with rapidly sedimenting membranes that can be extracted with high salt, and free vesicles that sediment in the HSP fraction. The smaller of the two Ypt1p containing particles is slightly smaller than secretory and clathrin-coated vesicles, consistent with the small (50nm diameter) size of ER-Golgi transport vesicles seen by electron microscopy.

Using an assay developed by Michael Rexach, a putative vesicular transport intermediate was found to accumulate in ypt1-1 mutant transport reactions and in wild type transport reactions containing anti Ypt1 Fab fragments. Michael has recently found that the Ypt1p requiring intermediate can be resolved from other membranes by velocity gradient sedimentation. Comparison of the sedimentation behavior of the intermediate and the smaller Ypt1p containing particle in the HSP fraction suggests that they may be similar objects. It is thus possible that the Ypt1p associated activity in the salt extract, the smaller particle in the HSP fraction and the Ypt1p requiring intermediate are all related. Functional Ypt1p on the surface of transport vesicles may be required for fusion with the Golgi. A mammalian homologue of Ypt1p, one of the Rab gene products, associates with the vesicular intermediate identified in the mammalian Golgi-Golgi transport reaction (Vivek Malholtra, personal communication).

Why should a transport reaction require supplementation with a transport intermediate? Perhaps the intermediate serves as a way-station like an endosome rather than being formed and consumed during each round of transport. Alternatively, a transport vesicle may

supply on its surface a peripherally associated limiting transport factor. Small vesicles could perhaps contain the bulk of the cellular pools of a clathrin-like coat protein or at least the bulk of the coat protein in an in vitro reaction. In the mammalian Golgi-Golgi transport reaction, supplementation with coated vesicles restores transport to reactions lacking a high molecular weight activity ("α") present in membrane salt extracts (Vivek Malholtra, personal communication).

Further experimentation is required to determine the relationship between the Ypt1p associated activity in the HSP fraction, the Ypt1p associated activity in the salt extract, and the slowly sedimenting Ypt1p requiring transport intermediate. The Ypt1 antibodies allow rapid purification of the Ypt1 associated particles. If the transport intermediate is similar to the activity in either the HSP or the salt extract, it should be possible to immunoprecipitate vesicles containing core-glycosylated α factor from Ypt1 inhibited in vitro reactions. Electron microscopic examination of the immunoprecipitates formed during incubations of each of the three fractions with protein A Sepharose beads coated with Ypt1 antibodies may allow a detailed morphological analysis of the Ypt1p associated particles.

Table 1 Cytosol and salt extract fractions are required for transport

Addition	anti α1-6Man precipitable cpm
--	61
50ug cytosol	82
15ug salt extract	146
50ug cytosol + 15ug salt extract	925

In a two-stage reaction, pro-α-factor-containing microsomes were prepared as described in chapter 3 and then incubated with the additions indicated above in 25ul reaction buffer containing GDP-mannose and an ATP regenerating system. Reactions were terminated after 1.5hr by addition of 2% SDS and reaction products were analyzed by immunoprecipitation followed by scintillation counting.

Table 2 Rescue of immunodepleted reactions

Addition	anti 1,6 Man precipitable cpm (% maximum)
PI treated cytosol, PI treated salt extract	100
αYpt1 treated cytosol, αYpt1 treated salt extract	2
+ cytosol (45ug)	52
+ cytosol (90ug)	80
+ HSP (1ug)	60
+ HSP (3ug)	95
+ salt extract (5ug)	23
+ salt extract (10ug)	90
+ 20ug salt extract--100000x g, supernatant	95
+ 20ug salt extract--300000x g, supernatant	15
+ 20ug salt extract--300000x g, pellet	80
+ recombinant Ypt1p (0.5ug)	2

Transport reactions prepared as described in table 1 were supplemented as indicated above. For differential centrifugation, 50ul of salt extract (10mg/ml) was centrifuged for 10min at 100,000xg or 15min at 300,000xg in a TLA 100 table top ultracentrifuge and the pellets resuspended in 50ul of reaction buffer. The first centrifugation should sediment particles of greater than 90S, the second, particles of greater than 12S. 2ul of either the supernatant or pellet fraction was added to transport reactions as indicated. PI=preimmune beads; αYpt1=anti Ypt1 beads.

Table 3 Ypt1 defective reactions accumulate a putative transport intermediate

Inhibitor	anti a1-6 linked Man precipitable cpm (% maximum)	Con A precipitable cpm in 12,000xg sup (% maximum)
A. ypt1-1 mutation		
no cytosol	10	20
ypt1-1 cytosol	25	100
wt cytosol	100	77
B. Ypt1 antibody		
no cytosol	4	12
wt cytosol	89	100
wt cytosol + Ypt1 Fab	25	90
wt cytosol + Ypt1 Fab + YPT1p	100	91

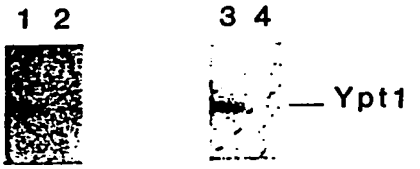
A. Transport reactions with ypt1-1 gently lysed cells were supplemented with ypt1-1 or wild type (wt) cytosol as indicated. After 30 min one set of reactions was centrifuged for 30 sec at 12,000x g and the supernatant fraction treated with Con A Sepharose. A second set of reactions was allowed to proceed for 60 min and then precipitated with anti α1,6 linked mannose antibodies.

B. Transport reactions with wild type microsomes were supplemented with cytosol, 0.5ug Ypt1 Fab fragments or 0.5ug recombinant Ypt1p as indicated. Two sets of reactions were carried out as described in A.

Figure 1. Immunodepletion of Ypt1p blocks transport

A. Salt extract (lanes 1 and 2) or cytosol (lanes 3 and 4) fractions were treated with preimmune serum (lanes 1 and 3) or Ypt1 antibodies (lanes 2 and 4) crosslinked to protein A Sepharose beads as described in Materials and Methods and then analyzed by SDS-PAGE followed by immunoblotting.

B. Standard microsome based transport reactions were supplemented as indicated with salt extract and cytosol fractions treated with preimmune (PI) or Ypt1 antibodies (YPT1 AB) as described in Material and Methods.


1 2
3 4
Ypt1

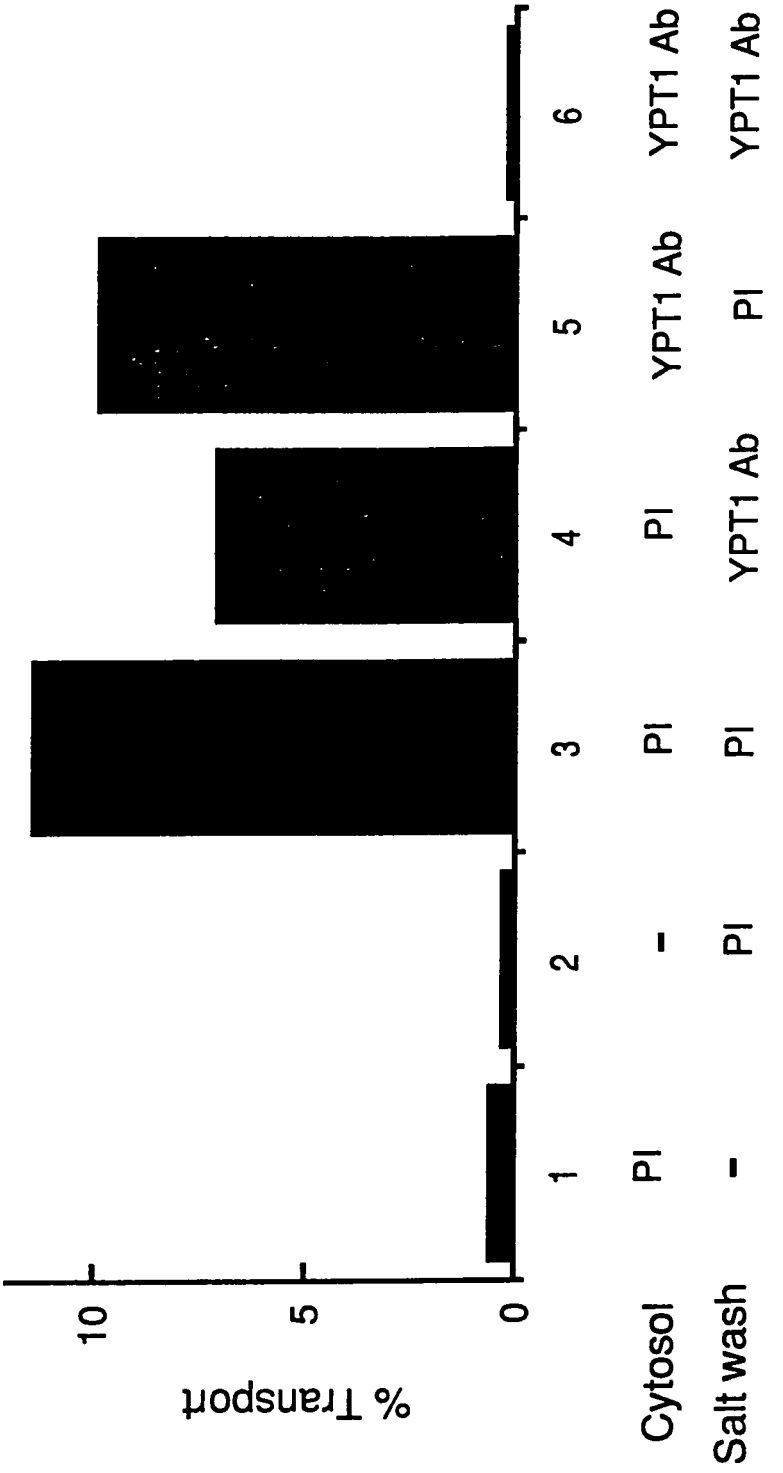
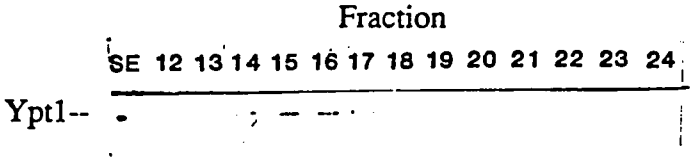

% Transport
0
5
10
1
2
3
4
5
6
Cytosol
PI
-
PI
YPT1 Ab
PI
YPT1 Ab
Salt wash
-
PI
PI
YPT1 Ab
PI
YPT1 Ab

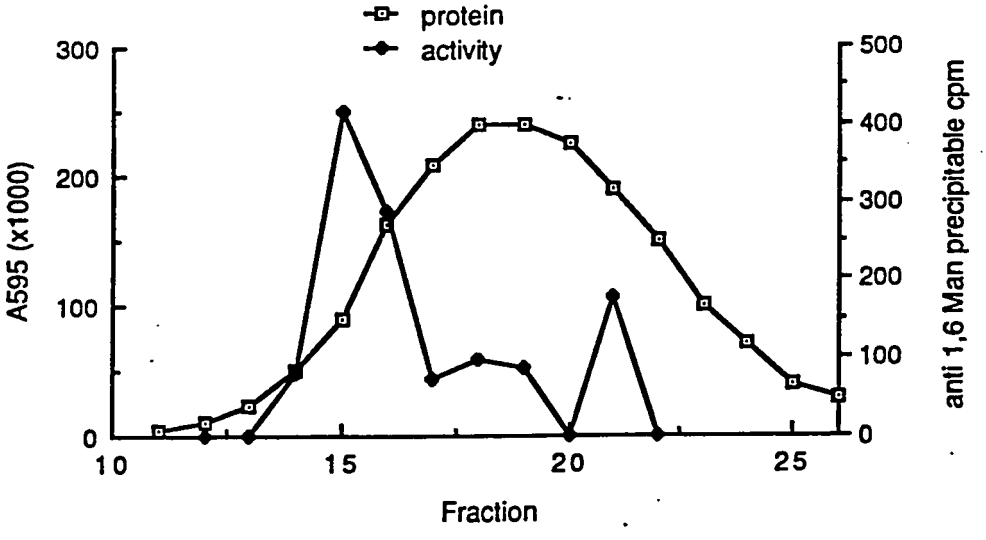
Figure 2. Sephacryl S500 fractionation of salt extract

Salt extract (150ul) were applied to a 7ml (0.7cm radius) Sephacryl S500 column equlibrated with reaction buffer and phospholipids. Fractions were subjected to immunoblotting with Ypt1 antibodies (A) and assayed for ability to restore transport activity to Ypt1p depleted transport reactions prepared as described in table 1 (B).

A. Ypt1 immunoblot



B. Activity profile



Appendix 4

Computer program for determining free ion concentrations in complex buffers

A Fortran IV program for determining the free cation concentrations in buffers containing arbitrary amounts of EGTA, ATP, K^+, Mg^{2+},Ca^{2+} and Mn^{2+} is listed on the next three pages. The program, which is adapted from Robertson and Potter (1984), uses a straightforward iterative procedure to solve the multiple coupled equilibria.

A note to the user: the program gives reasonably accurate results (estimated error < 5%) after 100 iterations even in the absence of series convergence.

```
C       Program Table
C
        REAL K,KO,MG,MGO,MN,MNO
C
C DEFAULT VALUES FOR ASSAY COMPOSITION AND CONDITIONS
C
        TEGTA = 0.005
        TATP  = 0.001
        TK    = 0.15
        TMG   = 0.005
        TMN   = 0.0001
        TEMP  = 20.0
        PH    = 6.8
        SINC  = 0.12
C
C CALCULATE EGTA COEFFICIENTS EH, EC, EM
C
        SHE  = SADJ(TEMP, 9.47, -5.8, 20.0) + SINC
        SH2E = SADJ(TEMP, 8.85, -5.8, 20.0) + SINC
        SH3E = 2.66 + SINC
        SH4E = 2.00 + SINC
        EH = 1.0 + (((10.0**(SH4E-PH) + 1.0)*10.0**(SH3E-PH) + 1.0)
     1    *10.0**(SH2E-PH) + 1.0)*10.0**(SHE-PH)
        SCE  = SADJ(TEMP, 10.97, -8.1, 20.0)
        SCHE = 5.29 + SINC
        EC = 10.0**SCE + 10.0**(SCHE+SHE-PH)
        SME = SADJ(TEMP, 5.21, 5.0, 20.0)
        SMHE = 3.36 + SINC
        EM = 10.0**SME + 10.0**(SMHE+SHE-PH)
        SNE = SADJ(TEMP, 12.11, -8.16, 20.0)
        SNHE = 6.59 + SINC
        EN = 10.0**SNE + 10.0**(SNHE+SHE-PH)
C
C CALCULATE ATP COEFFICIENTS AH, AC, AM, AK
C
        SHA  = 6.51 + SINC
        SH2A = 4.06 + SINC
        AH = 1.0 + (10.0**(SH2A-PH) + 1.0)*10.0**(SHA-PH)
        SCA = 3.77
        SCHA = 1.95 + SINC
        AC = 10.0**SCA + 10.0**(SMHA+SHA-PH)
        SMA = 4.06
        SMHA = 2.1 + SINC
        AM = 10.0**SMA + 10.0**(SMHA+SHA-PH)
        AK = 10.0**1.0
        SNA = 4.78
        SNHA = 1.37 + SINC
        AN = 10.0**SNA + 10.0**(SNHA+SHA-PH)
C
C OUTPUT ASSAY CONDITIONS AND SETUP HEADING
C
 10     WRITE(6,11) TEGTA, TK, TATP, TMN, PH, TEMP
 11     FORMAT(1H0, 31X, 'FINAL ASSAY CONDITIONS' /
     1  19X, 'ANION TOTALS', 24X, 'CATION TOTALS' /
     2  17X, 'EGTA', 1PE12.2, 23X, 'K', E12.2 /
     3  17X, 'ATP ', E12.2, 22X, 'MN', E12.2 //
     4  17X, 'ASSAY PH', 0PF8.2, 17X, 'TEMPERATURE', F8.1 //
     5  17X, 'PCA', 9X, 'FREE MN', 9X, 'FREE CA', 8X, 'TOTAL CA')
C
C CALCULATE TOTAL CA FROM PCA 2.0 TO 10.0 BY 0.1 PCA INCREMENTS
C
        DO 200 I=20, 100, 1
        PCA = FLOAT(I)/10.0
        CA = 10.0**(-PCA)
C
C START EACH CYCLE WITH [FREE CATION] = [TOTAL CATION]
```

```
C
        MG   = TMG
        K    = TK
        MN   = TMN
        ITER = 0
C
C CALCULATE [FREE ANION] USEING CURRENT [FREE CATION] VALUES
C
110     E = TEGTA/(EH + CA*EC + MG*EM + MN*EN)
        A = TATP/(AH + K*AK + CA*AC + MG*AM + MN*EN)
C
C SAME OLD VALUE OF EACH [FREE CATION] BEING CALCULATED
C
        MNO = MN
        MGO = MG
        KO  = K
C
C CALCULATE NEW [FREE CATION] VALUES
C
        MG  = TMG/(1.0 + E*EM + A*AM)
        MN  = TMN/(1.0 + E*EN + A*AN)
        K   = TK/(1.0 + A*AK)
        TCA = CA*(1.0 + E*EC + A*AC)
C
C INCREMENT COUNTER 'ITER' & CHECK ITS VALUE
C
        ITER = ITER + 1
        IF(ITER .GT. 1500) GO TO 190
C
C CHECK TO SEE IF EACH CALCULATED [FREE CATION] VALUE HAS CONVERGED
C
        Z = MG - MGO
        IF(MG .EQ. 0.0) GO TO 170
        Z = Z/MG
170     IF(ABS(Z) .GT. 0.0005) GO TO 110
C
        Z = K - KO
        IF(K .EQ. 0.0) GO TO 180
        Z = Z/K
180     IF(ABS(Z) .GT. 0.0005) GO TO 110
        Z = MN -MO
        IF (MN .EQ. 0.0) GO TO 185
        Z = Z/MN
185     IF (ABS(Z) .GT. 0.0005) GO TO 110
        GO TO 200
C
190     WRITE(6,191)
191     FORMAT('ITERATION LIMIT EXCEEDED. VALUES HAVE NOT CONVERGED')
C
200     WRITE(6,201) PCA, MN, CA, TCA
201     FORMAT(F21.2, 1P3E16.3)
        STOP
        END
C
C ADJUST STABILITY CONSTANT BASED UPON ASSAY TEMPERATURE
C
C       TEMP    ASSAY TEMPERATURE
C       PKA     LOG10 OF THE STABILITY CONSTANT
C       DH      ENTHALPY CHANGE PER DEGREE C
C       T       TEMPERATURE AT WHICH PKA AND DH WERE DETERMINED
C
        FUNCTION SADJ(TEMP, PKA, DH, T)
C
        DATA X/2.3025851/
        DATA R/1.96985E-3/
C
```

```
SADJ = PKA + DH*(TEMP-T)/(X*R*(T+273.2)**2)
RETURN
END
```

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